SPECTRUM ANALYSIS OF HEN EGGS AND CHICK TISSUES

W. F. DREA

Laboratory of the Colorado Foundation for Research in Tuberculosis, Colorado College, Colorado Springs

(Received for publication May 27, 1935)

There is an increasing amount of evidence for the biological importance of certain 'trace' elements.

The investigation of hen eggs and chick tissues was made to determine what elements passed from the rations and drinking water into the blood of the laying hen, hen eggs and tissues of chicks which had not ingested food or water.

The eggs and chicks were from a group of eggs laid at the time the water and rations investigated were being ingested by the laying hens. The blood of one laying hen was also secured at this time.

MATERIAL AND METHOD OF INVESTIGATION

The water was evaporated in silica and platinum dishes. The residue was used for analysis as there was very little organic material present. Ashing was not necessary.

Mash and a combination of wheat and corn were being fed to the hens at the time of investigation. The two kinds of feed and the yolks, whites and shells of five eggs were each ashed separately. This was done at low red heat in silica dishes. Each ash was separately analyzed. The blood of one of the laying hens was subjected to a similar procedure.

Three weeks later, ten chicks hatched in an incubator were used for the investigation of some of their tissues. The chicks had not ingested water or food. About 48 hours after being hatched, they were made unconscious by a sharp blow over

the top of the head. Sufficient feathers were removed from around the necks and the heads cut off. A few drops of the blood were allowed to escape, and the remainder was received in a platinum dish. The gizzard, kidney, brain, eye, lung, thigh muscle, liver, heart, blood and femur were separately ashed. The same method was used as for the eggs and feed. Most of the organs such as the kidneys, were so small that they had to be pooled. The blood of the chicks was also pooled. In addition, a femur from a 96-hour chick that had not ingested water or food was ashed.

No effort was made to burn off all the carbon in the substances which were resistant, and no reagents were added to promote ashing.

Precautions were taken to avoid contamination by extraneous elements. Spectra of the electrodes used were secured to note the presence of any elements already in them. The shell of an egg taken from the oviduct of a hen was analyzed as a control to eliminate the question of possible contamination of the laid eggs. The ashing and water evaporation was done in a room free from air currents, where presumably the dust in the air had previously settled.

The technic of securing the spectra was in general as reported before by Drea ('34). A direct current of 7.5 amperes was used for excitation of the spectrum after 10 to 20 mg. of the ash were placed on top of the lower of the two Acheson graphite spectroscopic electrodes. Iron arc spectra were used for measuring the wave lengths of the most 'persistent lines' of the elements as given in the International Critical Tables for arc spectra. Not all of the elements can have their spectra excited by the arc. Fluorine was sought by means of the CaF_2 band. A Hilger medium-sized quartz spectrograph was used. Eastman type III-F and type IV-F spectroscopic plates had the spectra recorded upon them. At least three spectra were photographed for each substance investigated.

No attempt at quantitative determinations was made.

RESULTS

Elements are reported as present or absent according to whether or not their characteristic wave lengths are present in the spectra of the investigated substances. Elements reported as absent may have been present in too small quantities to be revealed with the technic employed although generally speaking the spectrographic method is extremely delicate.

DISCUSSION AND SUMMARY

A number of elements pass from the feed and/or water into the hen's blood, from there into the egg, and finally into all of the chick's tissues and chick's blood. These are aluminum, barium, calcium, copper, iron, magnesium, phosphorus, potassium, rubidium (?) silicon, sodium, strontium, titanium and vanadium. Manganese and zinc were each absent from one organ and the chick's blood. Of the 'trace' elements, aluminum, barium, copper, silicon, strontium, titanium and zinc were quite uniformly distributed among the tissues.

There is a group of 'trace' elements more concentrated in the hen's blood and/or egg than in the feed and it appears reasonable that these are of high physiological importance. These are barium, iron, strontium and vanadium. The importance of iron is of course well known. The selection of barium and strontium may be caused by the inability of the hen's organism to distinguish these elements from calcium. The selection of vanadium is more difficult to explain: it is present in 4 plus quantities in the blood of both chick and hen, in the femur, heart, kidney and lung, suggesting some function associated with the hematopoietic organs.

There is a group of three elements whose presence in the newly hatched chick is unnecessary or harmful. These are boron, fluorine and silver. Boron although present in the food and water was found only in the egg yolk. Fluorine was present in the water in a concentration of about 2 p.m.m. It was not present in a sufficiently large amount to be demonstrated in the eggs, bloods or soft tissues. It was present in

Trace elements

	DRINKING WATER	RATIONS	HEN'S BLOOD	YOLKS	WHITES	SHELLS	CHICK'S BLOOD	BRAIN	EYE	FEMUR 48-HOUR	FEMUR 96-HOUR	GIZZARD	HEART	KIDNEY	LIVER	LUNG	MUSCLE
Al	++++	+++	++	+++	++	+	++	+	++	++	++	++	+++	+++	++	+++	+++
Ba	+++	+++	++	++++	++++	++++	+++	++	++	++	++	++	+	++	++	++	+
B	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Cr	+	+	+	3 out of 5 +	2 out of 5 +	1 out of 5 +	—	++	++	—	+	—	+	+	—	+	+
Cu	+++	++++	+++	+++	+++	+++	+++	++++	+++	+++	++++	+++	++++	++++	++++	+++	+++
F	++++	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—
Fe	++	+++	++++	++++	+	+	++++	+	+	++	+	++	++++	++++	++++	++++	++++
Pb	+	++	+	4 out of 5 +	2 out of 5 +	+	+	+	—	—	+	+	—	+	+	+++	+
Mn	++	++++	+	+++	—	—	—	++	—	+	+	+++	++	+++	++++	+	+
Mo	+	+	+	1 out of 5 +	+	4 out of 5 +	—	+	+	—	—	+	—	+	++	—	—
Rb	—	++	++	+	1 out of 5 +	—	+++	+	?	?	?	+	+	++	+	+++	++++
Si	++++	+	+	+++	++	++	+	+	+	+	+	+	++	+	+	+	+
Ag	+	—	—	—	3 out of 5 +	2 out of 5 +	—	—	—	—	+	—	—	—	—	—	—
Sr	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++++	++++	+++
Ti	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	++	+
V	++	+++	++++	+++	+++	+++	++++	+++	+++	+++	++++	+	++++	++++	+++	++++	+++
Zn	—	+	+	++	—	3 out of 5 +	—	+	+	+	+	+	+	+	+	+	—

Larger amounts of elements Ca, Mg, P, K and Na present in the usual larger amounts for such organic substances.

one femur and therefore must have been present in the egg from which this femur was evolved. This element is most probably of no physiological importance in any amount and when present in drinking water to the amount of more than 1 p.m.m. has a toxic effect upon the developing enamel of children's teeth. Silver was present in the water. It was not demonstrated in the hen's blood, egg yolk, chick blood or tissues with the exception of one femur.

Chromium, lead and molybdenum were present in approximately equal amounts in the hen's blood and in the feed and water, but were not constantly present in the eggs. Although the hen tolerates these elements in her own blood stream, she attempts to reject them for the formation of her eggs. The presence of these elements is tolerated in the adult organism but their presence is not necessary for the functioning of the chick and may be harmful. When the chicks contained these elements, molybdenum was found principally in the liver, lead in the lungs and chromium in the brain and eye. While we expect the accumulation of harmful heavy metals in the liver, the accumulation of chromium in the brain, and lead in the lung is surprising and at present unexplained.

Manganese was present in greater quantity in the yolks as compared with the hen's blood and was absent from the egg white and egg shell. It was concentrated in the liver and appeared in slightly lesser amounts in the kidney and gizzard. Its absence from the chick's blood and its presence in all of the tissues with the exception of the eye indicates a probable inadequate supply of this element which is of general nutritive importance.

LITERATURE CITED

DREA, W. F. 1934 Spectrum analysis of milk ashes. J. Nutrition, vol. 8, p. 229.

THE BASAL HEAT PRODUCTION OF THE RHESUS MONKEY (MACACA MULATTA)

NATHAN RAKIETEN[1]

Laboratory of Physiology, Yale University School of Medicine, New Haven

(Received for publication May 29, 1935)

The gaseous metabolism of the primates has only recently been the subject of investigation. Botschkareff ('33) determined the heat production of one macaque and three mandrills, but he was unable to secure basal conditions during observations. The only other published data are those of Bruhn ('34), who studied the basal metabolic rate of various species of primates including in his series four normal adolescent rhesus monkeys (Macaca mulatta). In view of the paucity of information concerning the metabolism of the rhesus monkey it has seemed worth while to record further studies on the basal heat production in this form.

METHODS

Six female and five male monkeys were studied. They were normal adolescent animals the weights of which varied from 2.7 to 3.6 kilos. The animals were generally fasted 18 to 24 hours prior to observation, but in one series the post-absorptive period was lengthened to 36 hours.

The determinations were made in an open circuit system modelled after the apparatus devised at the Carnegie Nutrition Laboratory at Boston (Carpenter and Fox, '31). The animal, confined in a small cage, was placed in a chamber of appropriate size through which outdoor air was circulated by

[1] When this work was done the author was a Porter Fellow of the American Physiological Society. The research was aided by a grant from the research funds of the Yale University School of Medicine. The author is at present in the Physiology Department at Massachusetts State College, Amherst, Massachusetts.

a rotary blower. The outgoing air after passing through two dry gas meters was collected for analysis by the Fox bag method of aliquot sampling (Carpenter and Fox, '31). The samples so obtained were stored in Benedict ('27) respiration pumps, and then analyzed in the Carpenter ('33) gas analysis apparatus. An activity recorder modified from that described by Benedict and Homans ('12) was used to register the animal's movements. Usually aliquots from four to five 20-minute periods were collected and the results of the two periods which gave the best agreement were taken to denote the animal's heat production. The lowest values were chosen in every instance. An agreement of 6 per cent or less was usually obtained for successive periods. Following the experiences of Bruhn ('34) the determinations were made at night to facilitate basal conditions.

The apparatus was tested intermittently during the investigation by burning alcohol in the chamber and by analyses of outdoor air. The mean respiratory quotient for alcohol in this series was 0.664 ± 0.003, while the greatest variations from the theoretical respiratory quotient of 0.667 were 0.674 and 0.658. The average results for the outdoor air analyses were 0.032 for CO_2 and 20.942 for O_2.

The surface area of the animals was calculated by the method of Lee and Fox ('33).

RESULTS AND DISCUSSION

The complete data obtained in these experiments are presented in table 1. It was found that the average heat production of the eleven animals was 608 Cal. per square meter per 24 hours. This is approximately 7 per cent lower than the average figures given by Bruhn ('34) for the four normal animals studied by him. Eight of the eleven animals had metabolic rates that ranged from 586 Cal. to 640 Cal. and the remaining three, numbers 30, 4 and 37, gave values, respectively, of 543, 543 and 709 Cal.

The wide divergences from the mean of these three animals were undoubtedly due to their distinctive metabolic rates.

Animal no. 37 which gave the highest value of the group was extremely excitable and it is conceivable that this high heat production may be accounted for by the emotional disturbances pointed out by Benedict ('35) and by the cardiac and respiratory factors which have recently been demonstrated by Carpenter et al. ('34) to produce a considerable augmentation in the O_2 consumption of normal individuals in the basal state. It is interesting to note that Bruhn ('34) obtained a value of 701 Cal. on one of the animals in his series.

TABLE 1

Results of metabolic rate determinations on eleven rhesus monkeys

ANIMAL NO.	NUMBER OF HOURS POST ABS.	SEX	WEIGHT IN KILOS	O_2 LITERS/ HR.	R. Q.	CALS./ SQ. M./ 24 HR.	CALS./ SQ. M./ HR.	CALS./ KILO/ 24 HR.
1	18	♀	3.7	1.570	0.753	640.0	26.7	48.7
2	18	♀	3.5	1.382	0.770	586.0	24.4	45.1
3	18	♀	3.6	1.506	0.745	628.0	26.1	48.5
30	24	♀	3.6	1.331	0.762	543.0	22.6	41.3
37	24	♀	3.5	1.680	0.745	709.0	29.5	54.4
24	36	♀	3.1	1.330	0.705	601.0	25.0	49.1
Average of females:						618.0		
4	18	♂	3.1	1.174	0.794	543.0	22.6	43.6
45	18	♂	2.9	1.248	0.742	595.0	24.8	48.9
46	18	♂	2.8	1.296	0.737	632.0	26.3	52.2
44	24	♂	2.7	1.206	0.724	600.0	25.0	50.5
23	24	♂	2.9	1.309	0.721	622.0	25.9	51.0
Average of males:						598.0		
Total average of entire group:						608.0		

Further evidence that this result is valid is supplied by two experiments in which the animal was given a subanesthetic dose of sodium amytal, 35 mg./kilo, a drug which has been demonstrated by many investigators (Anderson et al., '30; Deuel et al., '26; and Siebert and Thurston, '32), to produce little or no alteration of the basal metabolic rate even in anesthetic quantities. The findings in these experiments are presented in table 2. The average heat production in the two types of procedure was almost identical, the difference being approximately 1 Cal. Similarly the low metabolic rates of

animals nos. 30 and 4, both of which gave values of 543 Cal., must be attributed to their individual differences. It was surprising to find two animals of different weight and sex that had the same low basal metabolic rate. It is perhaps unwise to use the average metabolism of the group.

The trend of similar metabolic determination for the two sexes was noted throughout the investigation. The average heat production of the female group was 3.2 per cent higher than the male, 618 Cal. as compared with 598 Cal., a difference, however, which is well within the experimental error. Moreover, if the values obtained on animal no. 37, a female, are excluded the average results for the female group becomes 600 Cal., a value closely approximating the heat production of the males. It is possible that when more data on the heat

TABLE 2

B. M. R. of monkey no. 37 which received 35 mg. amytal/kilo

	NUMBER OF HOURS POST ABS.	SEX	WEIGHT IN KILOS	O_2 LITERS/ HR.	R. Q.	CALS./ SQ. M./ 24 HR.	CALS./ SQ. M./ HR.	CALS./ KILO/ 24 HR.
No amytal	24	♀	3.5	1.680	0.745	709.0	29.5	54.4
Amytal	24	♀	3.65	1.702	0.748	710.0	29.6	54.7

production of this animal have been accumulated similar sex differences such as those observed in the human by Benedict and Talbot ('21) will be evident.

Respiratory quotients. The respiratory quotients of the ten animals which were 18 to 24 hours post-absorptive ranged from 0.72 to 0.79. The quotients of seven of these animals fell within the limits of 0.74 and 0.77, while the three remaining animals nos. 4, 23 and 44, gave values of 0.79, 0.72 and 0.72, respectively. A series of determinations were carried out on animal no. 24 when it was 36 hours post-absorptive and its respiratory quotient at this time was 0.705. It would appear that the respiratory quotients of these animals decreases more rapidly than that of other mammals, a phenomenon that may possibly be associated with the monkey's high carbohydrate diet. The respiratory quotients obtained in this investigation

were slightly lower than those reported by Bruhn ('34), a difference that probably resulted from our use of a longer post-absorptive period.

SUMMARY

1. The average basal heat production of eleven adolescent rhesus monkeys was found to be 608 Cal./square meter/24 hours.

2. A similar heat production was observed for the males and females.

2. Two experiments on one animal which received 35 mg. of sodium amytal/kilo gave results almost identical with those obtained on it under normal basal conditions.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the interest and cooperation shown by Dr. J. F. Fulton all during this investigation. The author also wishes to express his gratitude to Dr. F. G. Benedict for his advice and aid during the study.

LITERATURE CITED

ANDERSON, H. H., M. Y. CHEN AND C. D. LEAKE 1930 Effects of barbituric acid hypnotics on basal metabolism in humans. J. Pharm. and Exp. Ther., vol. 40, p. 215.

BENEDICT, F. G., 1927 A 'field respiration apparatus' for a medical and physiological survey of racial metabolism. Bost. Med. Surg. J., vol. 197, p. 1161.

———— 1935 Degree of constancy in human basal metabolism. Am. J. Physiol., vol. 110, p. 521.

BENEDICT, F. G., AND J. HOMANS 1912 The metabolism of the hypophysectomized dog. J. Med. Res., vol. 25, p. 409.

BENEDICT, F. G., AND F. G. TALBOT 1921 Metabolism and growth from birth to puberty. Carnegie Inst. Wash. Pub. no. 302.

BOTSCHKAREFF, P. W. 1933 Arch. d. Sci. Biol., vol. 32, No. 1–2, p. 245. (Cited by Bruhn, '34).

BHUHN, J. M. 1934 The respiratory metabolism of infrahuman primates. Am. J. Physiol., vol. 110, p. 477.

CARPENTER, T. M. 1933 Ein Apparat zur Analyse von Gasen aus Respirationkammern für Menschen und Tiere. Abderhalden's Handbuch d. Biol. Arbeitsmethoden, Abt. IV, S. 593.

CARPENTER, T. M., AND E. L. FOX 1931 An apparatus for continuous short period measurements of human respiratory exchange during rest and muscular work. Arbeitsphysiol., vol. 4, p. 527.

CARPENTER, T. M., R. G. HOSKINS AND F. A. HITHCOCK 1934 Voluntarily induced increases in the rates of certain 'involuntary' physiological processes of a human subject. Am. J. Physiol., vol. 110, p. 320.

DEUEL, H. J., JR., W. H. CHAMBERS AND A. T. MILHORAT 1926 The influence of amytal upon the metabolism of the dog. J. Biol. Chem., vol. 69, p. 249.

LEE, M. O., AND E. L. FOX 1933 Surface area in a monkey, Macacus rhesus. Am. J. Physiol., vol. 106, p. 91.

SIEBERT, W. J., AND E. W. THURSTON 1932 Effect of amytal upon basal metabolism in guinea pigs. Proc. Soc. Exp. Biol. and Med., vol. 29, p. 650.

EFFECTS OF INCREASING THE CALCIUM CONTENT OF A DIET IN WHICH CALCIUM IS ONE OF THE LIMITING FACTORS

H. C. SHERMAN AND H. L. CAMPBELL

Department of Chemistry, Columbia University, New York City

TWO FIGURES

(Received for publication June 13, 1935)

It has been shown in previous papers (Sherman and Campbell, '24, '28, '30) that a diet adequate in the sense that it maintains normal growth and health with successful reproduction and lactation, generation after generation, may still be capable of improvement with resulting enhancement of nutritional well-being.

In the case of this kind which has been most extensively studied, the starting point was our diet A, a mixture of five-sixths ground whole wheat and one-sixth dried whole milk with sodium chloride and distilled water. At present writing (June, '35) our rat families are still thriving in the thirty-seventh generation on this diet. Notwithstanding this extraordinarily rigorous demonstration of the adequacy of diet A, it is not an optimal diet, for diet B (which differs from it only in containing a different quantitative proportion of the same articles of food) is clearly a better diet as shown by its influence upon growth and development, upon adult vitality, and upon length of life.

In chemical terms, diet B represents an enrichment of diet A in at least four factors: 1) protein (with presumably increased intake of histidine, lysine, tryptophane and perhaps other 'nutritionally essential' amino acids); 2) calcium; 3) vitamin A; 4) vitamin G (in the sense of the Bourquin-Sherman ('31) method) and perhaps the accompanying 'new

factor' (Booher, Blodgett and Page, '34). As this 'new factor' is relatively abundant in both milk and wheat (as well as in yeast, even after autoclave treatment) it must be contained in fairly liberal amounts in both diets A and B and may or may not play a part in the nutritional superiority of the latter. We are now engaged in somewhat extensive experimentation designed to determine which substances (in the chemical sense) are the 'limiting factors' in diet A—present in this food mixture in sufficient amount to permit of its being adequate, but below the optimal concentration as shown by the fact that diet A does not induce and support quite so high a state of nutritional well-being as does diet B.

The present paper records the results of our experiments with calcium as one of these limiting factors in an adequate but not optimal diet.

EXPERIMENTAL

The experiments here reported compare results with parallel lots or family groups of rats fed respectively upon laboratory diet 16 (the same as diet A) and laboratory diet 162 which differs from diet 16 in containing enough added calcium carbonate to give it the same calcium content as our diet B.

The initial lots or family groups of experimental animals each consisted of two males and three females. Parallel lots, made up when the rats were 4 weeks of age, were carefully matched as to heredity and size, and were kept strictly parallel as to all other conditions, and until the natural deaths of all the experimental animals. Inasmuch as more than one generation may be required for the full effect of a difference of diet, we have included also in this comparison parallel lots of offspring of the original matched lots in the second, third and fourth generations, these being likewise kept in lots of two males and three females each. The total numbers included in the experiments reported were: fifty-four males, seventy-seven females on diet 16; forty-nine males, seventy-two females on diet 162.

Many different numerical findings in the records of the life histories of the animals combine to serve as criteria of nutritional well-being. For the sake of conciseness these may be grouped under the three headings of, 1) rate and efficiency of growth, 2) indications of adult vitality, and 3) length of life.

Rate and efficiency of growth. The general average differences in growth and resulting size are shown in figure 1.

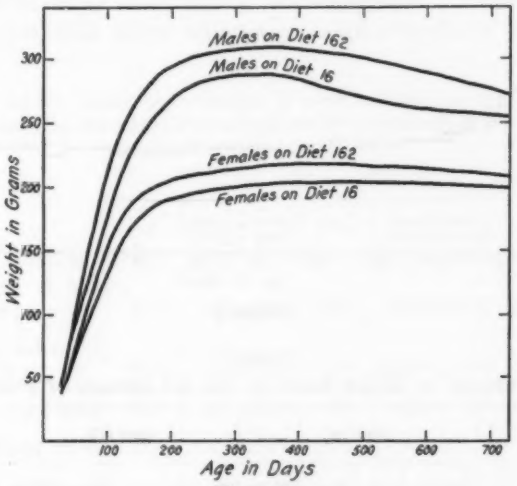


Figure 1

In each sex, growth was somewhat more rapid, and average size at a given age was somewhat greater, on the diet of more liberal calcium content (diet 162). Food consumption, per gram of body weight, was slightly higher on the same diet in the earlier stages of growth, but thereafter the food consumptions were essentially alike, so that in the general result the diet of higher calcium content was somewhat more efficiently utilized for growth and maintenance. The average ad libitum consumption of the two diets, in terms of calorie intake per 100 gm. of rat at different ages, is shown in figure 2.

A more precise quantitative comparison of the rate and efficiency of growth on the two diets during the fifth to eighth weeks, inclusive, of the lives of the rats is summarized in table 1.

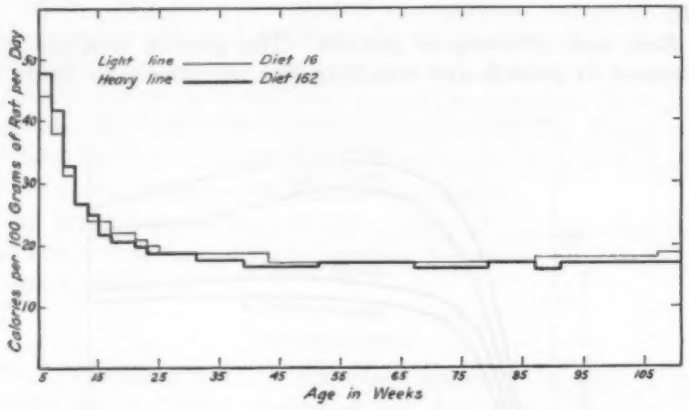


Figure 2

TABLE 1

Influence of calcium intake on rate and efficiency of growth

	DIET 16			DIET 162			DIFFERENCE WITH ITS PROBABLE ERROR
	Number of cases	C.V.[1]	Mean with its probable error	Number of cases	C.V.[1]	Mean with its probable error	
			grams			grams	
Gain in weight during 5th to 8th week, incl.							
Males	54	21.6	52.7 ±1.01	49	23.0	67.8 ±1.07	15.1 ±1.47
Females	77	24.1	46.2 ±0.82	72	18.5	59.0 ±0.85	12.8 ±1.18
Gain in weight per 1000 cal. consumed	131	13.3	64.9 ±1.06	121	11.3	72.7 ±1.00	7.8 ±1.46
Gain in weight per gram of protein consumed	131	13.4	1.83±0.030	121	11.5	2.06±0.029	0.23±0.041

[1] Coefficient of variation.

Thus growth during the period uncomplicated by puberty proves, on statistical analysis of the data from groups of from 49 to 131 cases, to have been significantly more rapid on the more liberal calcium intake and also significantly more efficient both on the basis of gain per 1000 cal. and on that of gain per gram of protein consumed. It should, however, be carefully noted that we are here dealing with only very moderate rates of growth, so that the higher of these seems well within the range of what is certainly advantageous. These experiments do not deal either with very rapid growth or with very high calcium intake. Within the zone with which these experiments are concerned, the more liberal calcium intake clearly induced a more favorable nutritional response.

TABLE 2

Influence of calcium intake on indications of adult vitality (breeding records of same females whose growth is shown in table 1 and fig. 1)

	DIET 16		DIET 162		DIFFERENCE WITH ITS PROBABLE ERROR
	C.V.	Mean with its probable error	C.V.	Mean with its probable error	
Age at birth of first young, days	25.3	137.3±2.79	22.9	115.9± 2.15	21.4± 3.52
Duration of ability to reproduce, days	65.6	184.9±9.24	57.2	235.3±10.56	50.4±14.0
Number of young born	68.8	20.7±1.10	63.7	25.5± 1.27	4.8± 1.68
Number of young reared	102.8	9.2±0.70	96.3	14.7± 1.11	5.5± 1.31
Weights of young of both sexes at the age of 28 days	9.8	37.2±0.09	9.4	40.1± 0.08	2.9± 0.12

Indications of adult vitality. The appearance and behavior of our animals indicate that, as between the two diets here compared, the more liberal calcium intake resulted in a higher average of adult vitality and its maintenance over a longer time. The chief objective numerical data yielded by our type of experiment are those of the breeding records of the females. These are summarized in table 2. These findings show that

the females on the more liberal calcium intake (diet 162) matured slightly earlier, showed a longer period of ability to reproduce, bore more young and reared a higher percentage of them, and to a slightly larger average weight at weaning. Inasmuch as none of these rats reached weights which could be regarded as excessive at any age, the slightly larger average size of the parents as well as the young may probably be interpreted as further evidence of superior nutritional well-being upon the higher of the two levels of calcium intake with which these experiments deal. Again, it may be emphasized that both these are moderate levels of intake, the lower is high enough to be within the range of adequacy as ordinarily understood, and the higher is by no means radically high.

TABLE 3

Lengths of life on diets of different calcium content

	DIET 16			DIET 162		
	Number of cases	C.V.	Mean with its probable error	Number of cases	C.V.	Mean with its probable error
			days			*days*
Males	54	24.4	651±14	49	19.7	705±13
Females	77	27.1	716±15	72	27.2	721±15

Length of life. The influence of added calcium upon length of life is less clear cut. Both males and females show longer average life cycles upon the higher of the two levels of calcium intake here studied. In the cases of the males the difference is very distinct, covering a large part of the difference previously found between diets A and B. In the case of the females the difference is small, and would not be regarded as significant if it stood alone. Viewed in the light of all the other data, including the fact that the females on diet 162 had reared significantly more young than those on diet 16, it is probable that for females as well as for males the more liberal calcium intake served to increase the length of life. The average lengths of life are shown in table 3. If in future it should prove possible, as we hope, to add materially to the number of these experiments upon length of life, the increased volume

of data will doubtless reduce the probable error of the means and thus permit a more conclusive evaluation of the differences.

Regularity of nutritional response. Together tables 1, 2 and 3 include coefficients of variability for the data of nutritional response or well-being, as shown on the two diets when compared in eleven different ways. In nine of the eleven comparisons the data for the animals on diet 162 show lower coefficient of variation (C.V.), i.e., the more liberal intake of calcium induced greater regularity of results as well as average outcomes indicative of better nutritional status.

DISCUSSION

In previous work with rats of the same strain, fed upon the same or similar diets (Sherman and MacLeod, '25; Sherman and Booher, '31), it has been shown that the present diet A or diet 16, while supporting growth and development at a rate within the normal range, does not induce as rapid a retention of calcium in the growing organism as does a calcium intake such as that of our diet 162; and on the level of intake afforded by diet 16 (about 0.2 per cent of calcium in the dry solids of the food mixture) the body requires a relatively long time to attain the same calcium content as that attained at the same age when the calcium intake is higher. The status supported by diet 16 is, however, well within the range of normal as this term is regularly used. It is only on much lower levels of intake, with only about half as much calcium in percentage of the food solids, that we have found evidence of subnormality attributable to low calcium content in the adult rat (Campbell, Bessey and Sherman, '35). It is also of interest to note that the calcium content of diet 16, whether calculated in percentage of the food solids or in proportion to the food calories, is higher in diet 16 than in average American family food supplies. There seems to be no room for doubt that, while the average American intake of calcium is adequate (in the accepted meaning of this word), it is below that from which optimal nutritional well-being is to be expected.

SUMMARY AND CONCLUSION

As part of an investigation to explain the improvement of an already adequate diet, the effect of such addition of calcium carbonate as to increase the calcium content of the dry food mixture from 0.2 per cent to 0.35 per cent has been studied with rats throughout the natural life cycle and through successive generations.

This enrichment of calcium intake was followed by a more efficient utilization of the food (whether calculated on the basis of its energy value or protein content); better growth; earlier maturity; several indications of higher adult vitality; a longer period between the attainment of maturity and the onset of senility; and, in less degree, an increase in the average length of adult life, or life expectation of the adult.

Inasmuch as the infant mortality was decreased by the increase in calcium intake, the life expectation at birth was increased in greater degree than the life expectation of the adult.

That the increase of the adult life cycle was not so large with the females as with the males may be due to the fact that the females on the higher calcium intake had borne and suckled more young.

The increase in the calcium content of the already adequate diet clearly improved its nutritive value as judged by the combined criteria of growth, adult vitality, lowered death rates, and increased length of life.

We conclude that the previously reported improvement of an already adequate food supply is in part (though not solely) attributable to the enrichment of the calcium intake, and that the level of calcium intake most conducive to optimal well-being is significantly higher than that required for normal growth and maintenance.

The cooperation of the Carnegie Corporation of New York and the Carnegie Institution of Washington is gratefully acknowledged.

LITERATURE CITED

BOOHER, L. E., H. M. BLODGETT AND J. W. PAGE 1934 Investigation of the growth-promoting properties of vitamin G concentrates. J. Biol. Chem., vol. 107, p. 599.

BOURQUIN, A., AND H. C. SHERMAN 1931 Quantitative determination of vitamin G (B_1). J. Am. Chem. Soc., vol. 53, p. 3501.

CAMPBELL, H. L., O. A. BESSEY AND H. C. SHERMAN 1935 Adult rats of low calcium content. J. Biol. Chem., vol. 110, p. 703.

SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. J. Biol. Chem., vol. 93, p. 93.

SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. J. Biol. Chem., vol. 60, p. 5.

———— 1928 The influence of food upon longevity. Proc. National Acad. Sci., vol. 14, p. 852.

———— 1930 Further experiments on the influence of food upon longevity. J. Nutrition, vol. 2, p. 415.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth, and food. J. Biol. Chem., vol. 74, p. 429.

CALCIUM AND PHOSPHORUS NEEDS OF PRESCHOOL CHILDREN

AMY L. DANIELS, MARY K. HUTTON, ELIZABETH M. KNOTT, OLIVE E. WRIGHT AND MARY FORMAN[1]

Iowa Child Welfare Research Station, State University of Iowa, Iowa City

FOUR FIGURES

(Received for publication June 12, 1935)

In a recent study of the calcium needs of children of preschool age, it was shown that under the conditions of the investigation the average calcium retentions were the same whether the children were receiving 1 pint or 1 quart of milk (Daniels et al., '34). These findings were so out of harmony with the generally accepted hypothesis that 1 quart of milk is necessary to supply the calcium for children of the ages considered (Sherman and Hawley, '22) that the work has been continued with certain slight modifications in technics and data alignment.

In calcium balance studies with children it is obvious that the relation of calcium furnished by a given quantity of milk to the total calcium of the diet differs with individuals, since in any group of children, even within a comparatively narrow age range, there will be more or less variation in size and therefore in the amount of foods other than milk taken. The calcium furnished by these foods, more particularly the fruits and vegetables, according to the findings of McClugage and Mendel ('18) and Sherman and Hawley ('22) is less available than that of milk although Rose ('20), Blatherwick and Long ('22), and Mallon, Johnson and Darby ('33) working with

[1] We are indebted to Miss Gladys Everson and Miss Florence Scoular for calculation of the diets and preparation of the meals.

adults, found the calcium of the vegetables tested equally well utilized. With few exceptions, the diets used for the children studied consisted of the same varieties of vegetables (carrots, tomatoes and potatoes) and fruits (prunes, apples, orange juice and banana) in very much the same proportions, therefore the availability of the calcium from these sources should be the same at a given calcium ingestion level. Variations in retentions under these conditions would seem to be due to the fact that some children needed more, either because of previous depletion or differences in potentialities of growth, or because other factors in the diet were influencing retentions, for example, an excess of calcium in relation to phosphorus or vice versa (Sherman and Pappenheimer, '21; McCollum, Simmonds, Parsons, Shipley and Park, '21) or insufficient vitamin D (Daniels, Stearns and Hutton, '29). In the study, too little vitamin D would seem to be ruled out since each child received twice daily, between breakfast and dinner and between dinner and supper, 3.5 cc. of a standard cod liver oil to which were added 4 drops of Viosterol. Furthermore, each child received daily a 10-minute exposure to the rays of a General Electric sun lamp. Both phosphorus and nitrogen ingestions and retentions have been included to determine to what extent these may have been influencing factors.

In order to meet the possible criticism of our first report that the preliminary periods were too short for metabolic adjustment, or that incomplete fecal eliminations or unavoidable inaccuracies in stool divisions may have introduced significant errors in the shorter metabolism periods, in the subsequent study the pre-periods were increased to 7 days, and two consecutive periods of 4 or 5 days each called 'double periods' were substituted for the single 5-day metabolism periods.

The food components of the diets were the same as those of the former study. In a few instances certain modifications in the amounts of given foods or in the choice of cereal foods were made for the purposes of another investigation. These apparently were without influence on the calcium and phosphorus retentions and therefore the data of these have been

included. In the interest of greater accuracy, the methods used in the preparation of the food were somewhat modified. These modifications are given in detail in the study of the protein needs of children (Daniels et al., '35). The food intakes were so adjusted that one-third of the fat, protein and carbohydrate was given at each of the three meals. In so far as possible, the milk ingestion was similarly apportioned.

The children, who were between 36 and 66 months of age were cared for as meticulously as those of the previous report. All were in good health during the period of the study, although all were not equally robust.

METHODS OF ANALYSIS

The analytical methods of the present study were the same as those used in the previous report (Daniels et al., '34) with the exception of a slight modification in the calcium determinations by which the calcium oxalate was precipitated at a slightly lower hydrogen-ion concentration according to the method of Washburn and Shear ('32). Tests of accuracy of the calcium and phosphorus methods by additions of known amounts to stool and food samples gave recoveries of 100.1 and 99.7 per cent, respectively. With the exception of duplicates of the urinary phosphorus, all determinations were made in triplicate.

Since retentions in the studies involving the somewhat more refined technics were found to be similar to those of the previous report, for purposes of statistical analysis the results of all calcium balance studies have been grouped according to calcium ingestions per kilogram of body weight at intervals of five between 35 mg. and 100 mg. with the corresponding phosphorus and nitrogen ingestions. The scatter diagrams showing the relation of calcium ingestions to retentions indicate the double periods as well as the first and second consecutive periods of the former study.

The higher calcium retentions of childhood are concerned chiefly with skeletal growth; thus it seemed possible that retentions in relation to ingestions based on height might give

more accurate information regarding children's needs than retentions based on weight. In the study, therefore, the retentions have been grouped also in relation to height between ingestions of 7.0 mg. and 14.9 mg. per centimeter at intervals of ten.

RESULTS

A consideration of the average calcium retentions at the various levels of ingestion tested (table 1) gives little information concerning the needs of children of the ages studied. With the exception of two cases at the upper level of ingestion, the highest average retention, 12 mg. per kilogram, was obtained with ingestions between 60 and 64 mg. per kilogram, whereas with ingestions between 90 mg. and 94 mg. per kilogram the average retention was somewhat less, 9 mg. per kilogram. It will be noted that the range of retentions is fairly comparable at each ingestion level above 40 to 44 mg.

Optimum retentions at a given ingestion level, which would seem to be indicative of the availability of the calcium, were not directly related to the ingestions, within the limits of the study. For example, retentions of 15 mg. per kilogram were obtained with ingestions of 45 mg., 65 mg., 85 mg., and 95 mg. per kilogram, respectively, while retentions of 14 mg. were found with ingestions of 70 mg. as well as 80 mg. per kilogram. At the ingestion level of 35 to 39 mg., both average and optimum retentions were low, but not lower than certain retentions obtained with higher ingestions. Whether these lower retentions were due to too little calcium in the diet or to the fact that the children needed less is not clear. This lower ingestion level included five balance studies with two children who were unusually robust and considerably (from 5.6 to 19.4 per cent) above the theoretical weight for height. Estimating the retentions of these children on the basis of their theoretical weights, brings the average well within the range of the groups above.

Calcium ingestions based on height (table 2) also seem to indicate that sufficient calcium was given at all levels of ingestion tested. The highest retention, 2.8 mg. per centimeter,

TABLE 1

The relation of calcium retention to calcium ingestion based on weights of the children studied

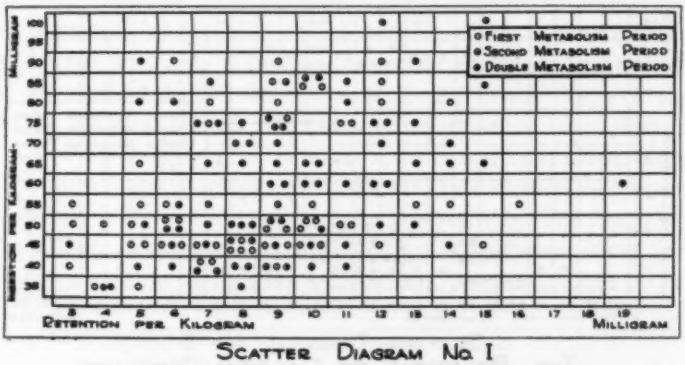
CALCIUM INGESTION PER KILOGRAM	NUMBER OF CASES	AVERAGE AGE	AVERAGE WEIGHT	CALCIUM RETENTION			PHOSPHORUS INGESTION		NITROGEN INGESTION	
				Average per kilogram	Standard deviation	Range per kilogram	Average per kilogram	Range per kilogram	Average per kilogram	Range per kilogram
mg.		*yr. mo.*	*kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
95–100	2	3– 8	13.57	14	..	12–15	94	91– 96	620	580–660
90– 94	5	4– 5	15.16	9	..	5–13	95	86–101	610	540–680
85– 89	10	4– 8	15.76	10	2	8–15	88	79–100	590	490–700
80– 84	7	4–11	16.69	9	3	5–14	81	75– 90	520	460–570
75– 79	13	5– 0	17.71	10	2	7–13	80	73– 89	570	470–610
70– 74	5	4– 9	17.80	10	..	8–14	79	74– 87	540	470–660
65– 69	8	4–11	18.05	10	3	5–15	73	63– 84	510	400–610
60– 64	8	4– 6	18.51	12	3	9–19	73	64– 78	540	470–630
55– 59	10	3– 6	13.85	9	4	3–16	70	63– 76	520	440–570
50– 54	21	4– 8	15.58	8	2	4–12	69	59– 82	520	420–630
45– 49	24	5– 1	17.22	9	2	5–15	62	53– 76	460	390–550
40– 44	12	5– 1	18.50	8	2	3–11	60	51– 67	460	390–530
35– 39	5	5– 7	22.50	5	..	4– 8	54	52– 59	420	390–430

TABLE 2

The relation of calcium retention to calcium ingestion based on heights of the children studied

CALCIUM INGESTION PER CENTIMETER	NUMBER OF CASES	AVERAGE AGE	AVERAGE HEIGHT	CALCIUM RETENTION			PHOSPHORUS INGESTION		NITROGEN INGESTION	
				Average per centimeter	Standard deviation	Range per centimeter	Average per centimeter	Range per centimeter	Average per centimeter	Range per centimeter
mg.		*yr. mo.*	*cm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
14.0–14.9	6	3– 9	93.6	1.8	0.36	1.2–2.2	13.6	13.0–14.0	90.5	81.5– 96.5
13.0–13.9	28	4–10	102.9	1.5	0.43	0.7–2.6	13.8	11.1–15.5	92.4	75.8–109.2
12.0–12.9	11	4–11	106.6	1.7	0.41	1.0–2.3	13.2	11.8–14.5	88.4	72.8–104.7
11.0–11.9	2	4– 8	104.7	1.8	....	1.8–1.9	13.5	13.4–13.6	100.3	98.6–101.9
10.0–10.9	8	4–10	104.6	2.0	0.39	1.5–2.7	12.1	9.9–13.4	87.8	64.9–109.2
9.0– 9.9	3	4– 2	100.1	2.1	....	1.7–2.8	12.0	11.4–12.8	82.7	74.8– 92.2
8.0– 8.9	30	4– 4	98.5	1.3	0.45	0.4–2.3	10.9	9.3–12.4	81.1	64.5– 98.5
7.0– 7.9	42	5– 1	103.9	1.3	0.40	0.6–2.4	10.3	9.1–12.4	77.1	62.8– 96.0

was obtained with an ingestion of 9.9 mg. per centimeter. At ingestions between 14.0 and 14.9 mg., and 11.0 to 11.9 mg., the same average retentions were obtained, namely, 1.8 mg. per centimeter, which was slightly lower than the average retentions of 2.0 and 2.1 obtained with ingestions between 10.0 and 10.9 mg. and 9.0 to 9.9 mg. per centimeter, respectively. The upper range of retentions at these ingestion levels was also quite similar, namely, 2.7 and 2.8 mg., respectively. With an ingestion of 14.0 to 14.9 mg., the highest of the series one child retained only 2.2 mg., slightly less than the optimum,

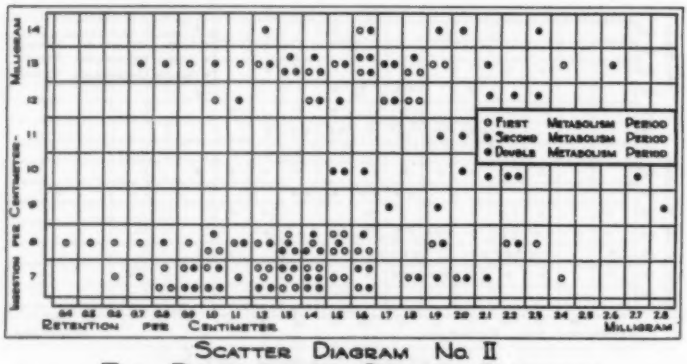


Scatter Diagram No. I
The Relation of Calcium Retention to Calcium Ingestion
Based on Weights of the Children Studied

2.4 mg. per centimeter, obtained at the lowest ingestion, 7.0 to 7.9 mg. per centimeter.

The scatter diagrams depicting the calcium retentions estimated on both weight and height (scatter diagrams 1 and 2) even more clearly than the tables, indicate that under the conditions of the study the calcium needs of children can be met by lower ingestions than we have hitherto considered necessary. Seventy-four per cent of all the children studied retained 10 mg. per kilogram or less. Of these 63 per cent received 1 pint of milk, and 37 per cent between a pint and a quart. Based on height, 69 per cent of the children studied retained

1.6 mg. per centimeter or less, irrespective of the amount ingested, 32 per cent of these being among those receiving 10 mg. or more per centimeter from diets containing more than a pint of milk, and 68 per cent among those receiving less than 10 mg. per centimeter. High retentions estimated either on weight or height are found at all ingestion levels. Among those receiving either a pint or a quart of milk are a number of children with high retentions, who were studied during several metabolism periods, although these were not consecutive. Estimating these high retentions, either on the



SCATTER DIAGRAM No. II
THE RELATION OF CALCIUM RETENTION TO CALCIUM INGESTION
BASED ON HEIGHTS OF THE CHILDREN STUDIED

basis of weight or height, of the individual children studied, brings the percentage of retentions above 10 mg. per kilogram, and 1.6 mg. per centimeter to 16 per cent and 18 per cent, respectively.

In a previous report regarding the protein needs of children (Daniels et al., '35) it was suggested that the wide variations in the nitrogen retentions at given ingestions above the minimum were due either to previous depletion of the children studied or to differences in potentialities of growth. The data of the present investigation suggest that calcium retentions also are related to the physical condition of the children studied as well as to the growth pattern. On the other hand,

in the diets of the children, there were more or less differences in the amounts of phosphorus and nitrogen ingested. Studies with animals have shown that any considerable disparity between the calcium and phosphorus ingestions, resulting in an excess of either, may materially influence the calcium retentions (Hart, Steenbock and Elvehjem, '24; Shohl et al., '33). The addition of vitamin D or exposure to the ultra-violet rays of the sun or of a mercury quartz lamp tend to nullify the results (Hart, Steenbock and Hoppert, '21; Templin and Steenbock, '33). Similarly, the untoward effects of a diet potentially acid or basic is modified by the inclusion of sufficient amounts of vitamin D (Morgan, '34). From experiments with animals, therefore, one may conclude tentatively that any imbalance of these two elements in the diets of the children studied would not have affected materially the results, provided, of course, enough of each were supplied, since the vitamin D given was controlled, and in so far as is known, adequate. That such was the case is shown by the fact that similar retentions were obtained at quite different ingestion levels of the three metabolites, calcium, phosphorus and nitrogen, which are conceded to be interrelated. Space allows for only a few examples at given ingestion levels (table 3).

Comparing the retentions resulting from dissimilar ingestion levels, it is obvious that the amounts and relationships of these metabolites were not the determining factors in the amount of calcium retained, since comparable retentions were obtained with very different ingestions. Nor can the availability of the calcium of the diet indicated by the percentage of calcium from milk be the determining factor.

It would seem that the needs of the children differed at the particular time of the study. Growth in childhood is a continuous, but not necessarily a symmetrical process. During a given period, some tissues may be growing at a more rapid rate than others and requiring different amounts of the substance tested, or previous to the study certain of the children may have received adequate amounts of some substances and less than the optimum amounts of others; thus the

TABLE 3

Similar retentions of calcium, phosphorus and nitrogen at different levels of ingestion

NAME	DATE	AGE	WEIGHT	PER CENT OVER + OR UNDER − THEORETICAL WEIGHT [1]	RETENTION PER KILOGRAM			RETENTION RATIO $\left(\frac{N}{P-\frac{99\%\ Ca}{1.94}}\right)$	INGESTION PER KILOGRAM			TOTAL CALCIUM FROM MILK
					Calcium	Phosphorus	Nitrogen		Calcium	Phosphorus	Nitrogen	
		yr. mo.	*kg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
B. S.	1/30/34	4– 5	15.41	— 8.0	14.3	10.8	70	20 : 1	72	83	570	85
L. B.	2/1/34	5– 2	16.59	— 3.7	14.2	11.1	76	19 : 1	69	79	580	85
L. B.	1/10/34	5– 1	16.39	— 2.1	13.5	10.8	96	25 : 1	47	55	410	80
F. W.	6/12/32	3– 1	14.04	+ 2.1	13.0	10.5	106	27 : 1	92	89	540	91
C. H.	11/14/34	4– 9	15.78	— 7.9	12.4	9.7	107	31 : 1	54	77	630	72
B. S.	2/21/34	4– 6	15.95	— 4.9	12.0	9.7	99	27 : 1	73	87	660	82
P. D.	5/2/34	4–10	17.09	+ 6.1	11.7	7.8	80	42 : 1	75	84	810	90
D. B.	11/14/32	4– 3	16.10	+ 0	11.3	7.6	79	44 : 1	51	65	470	73
D. B.	12/2/32	4– 4	17.00	+ 4.7	11.0	8.3	72	27 : 1	46	63	430	77
C. P.	6/11/33	6– 1	17.50	— 4.8	11.8	8.3	67	29 : 1	81	75	460	90
J. E.	10/1/34	3–11	15.78	+ 8.1	10.0	7.7	92	35 : 1	86	83	570	90
D. G.	11/14/34	4– 8	19.33	+ 7.4	10.5	7.7	80	39 : 1	43	63	550	73
G. O.	5/12/33	6– 1	16.65	— 8.4	9.6	9.1	115	27 : 1	87	100	690	84
C. P.	6/23/33	6– 2	17.50	— 4.8	9.2	9.1	114	25 : 1	80	86	570	89
N. L.	6/10/34	3– 2	14.52	— 0	8.5	6.2	80	42 : 1	54	76	570	77
L. T.	3/5/33	4–10	14.96	—12.0	8.7	6.2	76	42 : 1	54	73	590	75
A. C.	2/3/33	5– 5	18.00	+12.5	7.3	11.2	103	14 : 1	44	62	480	73
A. C.	3/19/33	5– 6	18.00	+ 9.1	7.4	11.0	112	15 : 1	44	60	450	77
R. H.	3/5/33	5– 0	18.30	+ 5.8	6.9	5.6	56	27 : 1	44	60	480	75
B. S.	10/4/33	4– 2	12.90	— 5.1	6.5	5.6	57	25 : 1	57	63	440	81

[1] Theoretical weight estimated from: 'Physical traits of young children.' Iowa Child Welfare Research Station, '29. Am. J. Dis. Child., vol. 38, p. 541.

relationship between calcium and phosphorus on the one hand, and phosphorus and nitrogen on the other would not be constant. When skeleton, muscle and glandular tissue are growing at the optimum rate as in infancy, during adolescence, or convalescence from a wasting disease, a fairly constant ratio between calcium, phosphorus and nitrogen would be expected. During periods of less rapid growth, somewhat different ratios would obtain. Since phosphorus is necessary for the development of bone as well as muscle and glandular tissue, it seemed that the phosphorus retention might be indicative of the nutritional status of the child, especially when it is considered in relation to the calcium and nitrogen retentions. The data of the study, therefore, have been grouped in relation to the phosphorus retentions at intervals of ten between 2.5 mg. and 14.4 mg. per kilogram, with the corresponding nitrogen and calcium retentions (table 4). The relation of phosphorus to nitrogen after the calcium needs for bone growth have been satisfied, called the retention ratio $\left(\frac{N}{P-\frac{99\%\ Ca}{1.94}}\right)$ also has been determined for each retention level.

At a given phosphorus retention level, these retention ratios were found to be surprisingly though not absolutely constant, averaging 17 : 1 at the highest retentions of 13.5 to 14.4 mg. per kilogram, and gradually increasing as the phosphorus retention decreased until at retentions of 3.5 to 4.4 mg. per kilogram the ratio was 34 : 1. At 2.5 to 3.4 mg. per kilogram, slightly less phosphorus than enough to combine with the calcium for bone formation was retained. These low phosphorus retentions were obtained with two unusually robust children who were considerably (29 per cent) above average weight.

The phosphorus ingestions, apparently, were not a factor in influencing these low retentions since high retentions were observed quite as frequently among children receiving low ingestions. This is shown by the scatter diagrams depicting the relation of phosphorus retentions to phosphorus ingestions. In general, low phosphorus retentions were coexistent

TABLE 4

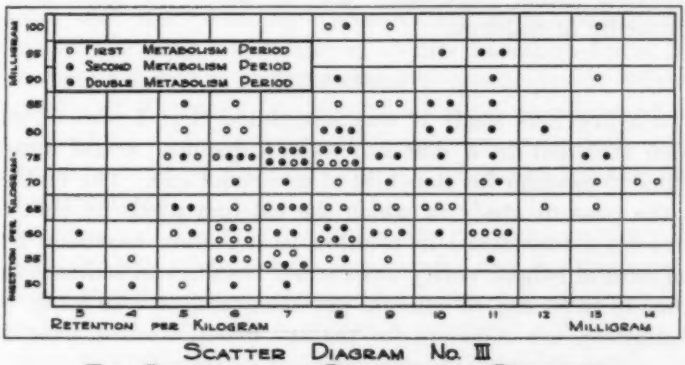
The interrelation of phosphorus, calcium and nitrogen retentions based on comparable phosphorus retentions

PHOSPHORUS RETENTION RANGE PER KILOGRAM	NUMBER OF CASES	AVERAGE AGE	AVERAGE WEIGHT	PER CENT OVER + OR UNDER − THEORETICAL WEIGHT [1]	AVERAGE RETENTION PER KILOGRAM			RETENTION RATIO $\left(\frac{N}{\frac{P - 99\%\ Ca}{1.94}}\right)$	AVERAGE INGESTION PER KILOGRAM		
					Phosphorus	Calcium	Nitrogen		Phosphorus	Calcium	Nitrogen
mg.		*yr. mo.*	*kg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
13.5–14.4	2	3– 7	15.29	+ 5.5	14.0	11.8	135	17 : 1	71	54	540
12.5–13.4	6	4– 0	14.73	— 2.4	13.2	13.0	108	16 : 1	80	67	530
11.5–12.4	2	4– 0	14.53	— 6.5	12.1	11.9	108	18 : 1	76	63	560
10.5–11.4	13	4– 8	15.81	— 0.7	11.0	11.6	97	19 : 1	76	66	530
9.5–10.4	12	4– 6	16.06	— 2.4	9.9	10.6	101	22 : 1	77	66	560
8.5– 9.4	12	4–10	17.13	+ 1.4	8.9	8.5	105	23 : 1	73	62	510
7.5– 8.4	24	4–11	17.12	— 1.0	7.9	8.8	92	27 : 1	73	63	520
6.5– 7.4	21	4–11	17.25	+ 2.2	6.9	8.3	77	29 : 1	67	51	490
5.5– 6.4	19	4– 8	16.86	+ 2.3	5.9	7.6	70	34 : 1	68	58	500
4.5– 5.4	10	4– 5	17.44	+ 4.9	5.1	7.3	64	46 : 1	72	67	500
3.5– 4.4	3	5– 4	20.70	+ 5.3	4.0	4.3	59	34 : 1	59	41	440
2.5– 3.4	2	5– 0	21.62	+ 29.2	3.0	6.6	47	....	58	50	430

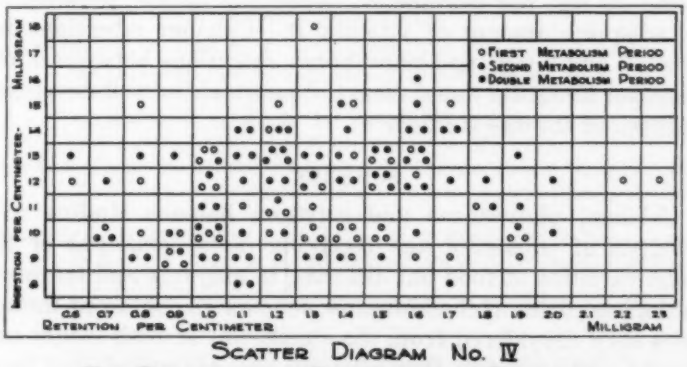
[1] Theoretical weight estimated from: 'Physical traits of young children.' Iowa Child Welfare Research Station, '29. Am. J. Dis. Child, vol. 38, p. 541.

with low nitrogen retentions, indicating that the soft tissues of these children were well developed.

Calcium retentions, on the other hand, with the exception of those at the higher levels, remained more nearly constant,



SCATTER DIAGRAM No. III
THE RELATION OF PHOSPHORUS RETENTION
TO PHOSPHORUS INGESTION
BASED ON WEIGHTS OF THE CHILDREN STUDIED



SCATTER DIAGRAM No. IV
THE RELATION OF PHOSPHORUS RETENTION
TO PHOSPHORUS INGESTION
BASED ON HEIGHTS OF THE CHILDREN STUDIED

between 6.6 and 8.8 mg. per kilogram, suggesting that the high retentions either of phosphorus or calcium are the result of previous depletion. Further evidence of this is suggested by the retention ratios obtained by Jeans and Stearns ('33)

during infancy, by Stearns and Moore ('31) in under-nutrition and subsequent recovery in a 3-year-old child, and by Lüthje and Berger ('04) during convalescence from typhus in an adult. Low phosphorus retentions, when coexistent with high retention ratios apparently are indicative of more nearly physically fit children.

COMMENT

In a previous study it was postulated that 1 pint of milk when included in a diet otherwise adequate would supply sufficient calcium for the child of preschool age. The findings in the present study in which the calcium needs of children have been considered in relation to ingestion based on both weight and height, verify our former results and indicate that from 45 to 50 mg. of calcium per kilogram, or 7 to 8 mg. per centimeter, are sufficient for the age group studied, provided the calcium is available and sufficient vitamin D is allowed.

The phosphorus needs of children of the age group studied seemingly can be met by 55 to 60 mg. per kilogram, or between 9 and 10 mg. per centimeter, depending on the presence of an adequate amount of vitamin D. Calcium retentions between 7 and 9 mg., and phosphorus retentions between 6 and 9 mg. per kilogram would seem to be normal for well-developed children of the age studied.

SUMMARY

Calcium, phosphorus and nitrogen balance studies with children of preschool age receiving diets composed of the same varieties of food but differing in amounts and therefore in the quantities of the inorganic substances contained therein have been considered from the standpoint of both height and weight. The vitamin D in all cases was controlled, and in so far as is known, adequate, thus ruling out any variation which might accrue from too little.

Wide variations in both calcium and phosphorus retentions were obtained at similar ingestion levels. High retentions were interpreted as being due to previous depletion.

The retention ratios of the nitrogen to phosphorus after the phosphorus needs for bone growth had been satisfied $\left(\frac{N}{P-\frac{99\%\ Ca}{1.94}}\right)$ grouped according to the phosphorus retentions were found to increase as the phosphorus retentions decreased and were roughly proportional to the nutritional status of the child as indicated by the variation from the theoretical weight for height of the group considered. Within the limits of the study, the retentions were uninfluenced by the ingestions. The majority of the calcium retentions were found to be between 6 and 10 mg. per kilogram, irrespective of the ingestions.

The results of the study indicate that the calcium needs of normal children of preschool age can be met by foods containing between 45 and 50 mg. of calcium per kilogram, or between 7 and 9 mg. per centimeter, provided sufficient vitamin D is allowed. Phosphorus needs, on the other hand, can be met by food containing between 60 and 70 mg. per kilogram, or between 9 and 11 mg. per centimeter.

LITERATURE CITED

Blatherwick, N. R., and M. L. Long 1922 The utilization of calcium and phosphorus of vegetables by man. J. Biol. Chem., vol. 52, p. 125.

Daniels, A. L., G. Stearns and M. K. Hutton 1929 Calcium and phosphorus metabolism in artificially fed infants. I. Influence of cod liver oil and irradiated milk. Am. J. Dis. Child., vol. 37, p. 296.

Daniels, A. L., M. K. Hutton, E. Knott, G. Everson and O. Wright 1934 The relation of the ingestion of milk to calcium metabolism in children. Am. J. Dis. Child., vol. 47, p. 499.

Daniels, A. L., M. K. Hutton, E. M. Knott, O. E. Wright, G. J. Everson and F. Scoular 1935 A study of the protein needs of preschool children. J. Nutrition, vol. 9, p. 91.

Hart, E. B., H. Steenbock and C. A. Hoppert 1921 Dietary factors influencing calcium assimilation. I. The comparative influence of green and dried plant tissue, cabbage, orange juice, and cod liver oil on calcium assimilation. J. Biol. Chem., vol. 48, p. 33.

Hart, E. B., H. Steenbock and C. A. Elvehjem 1924 Dietary factors influencing calcium assimilation. V. Effect of light upon calcium and phosphorus equilibrium in mature lactating animals. J. Biol. Chem., vol. 62, p. 117.

Jeans, P. C., and G. Stearns 1933 Growth and retentions of calcium, phosphorus and nitrogen of infants fed evaporated milk. Am. J. Dis. Child., vol. 46, p. 69.

LÜTHJE, H., AND C. BERGER 1904 In welcher Form kommt aus der Nahrung retinierter Stickstoff im Organismus zur Verwendung. Deut. Arch. Klin. Med., vol. 81, p. 278.

MALLON, M. G., L. M. JOHNSON AND C. R. DARBY 1933 The calcium retention on a diet containing leaf lettuce. J. Nutrition, vol. 6, p. 303.

MCCOLLUM, E. V., N. SIMMONDS, H. T. PARSONS, P. G. SHIPLEY AND E. A. PARK 1921 Studies on experimental rickets. I. The production of rachitis and similar diseases in the rat by deficient diets. J. Biol. Chem., vol. 45, p. 333.

MCCLUGAGE, H. B., AND L. B. MENDEL 1918 Experiments on the utilization of nitrogen, calcium, and magnesium in diets containing carrots and spinach. J. Biol. Chem., vol. 35, p. 353.

MORGAN, AGNES FAY 1934 The effect of acid, neutral and basic diets on the calcium and phosphorus metabolism of dogs. University of California Publications in Physiology, vol. 8, p. 61.

ROSE, M. S. 1920 Experiments on the utilization of the calcium of carrots by man. J. Biol. Chem., vol. 41, p. 349.

SHERMAN, H. C., AND A. M. PAPPENHEIMER 1921 Experimental rickets in rats. Diets producing rickets in white rats and its prevention by an addition of an inorganic salt. J. Exp. Med., vol. 34, p. 189.

SHERMAN, H. C., AND E. HAWLEY 1922 Calcium and phosphorus metabolism in childhood. J. Biol. Chem., vol. 53, p. 375.

SHOHL, A. T., H. B. BROWN, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN 1933 The evaluation of the phosphorus deficiency of the rickets-producing diet. J. Nutrition, vol. 6, p. 271.

STEARNS, G., AND D. L. R. MOORE 1931 Growth in height and weight and retention of nitrogen, calcium and phosphorus during recovery from severe malnutrition. Am. J. Dis. Child., vol. 42, p. 774.

TEMPLIN, V. M., AND H. STEENBOCK 1933 Vitamin D and the conservation of calcium in the adult. The effect of vitamin D on the calcium conservation in adult rats maintained on low calcium diets. J. Biol. Chem., vol. 100, p. 209.

WASHBURN, M. L., AND M. J. SHEAR 1932 Composition of bone. XIII. Direct gravimetric determination of Ca, Mg, and PO_4. J. Biol. Chem., vol. 99, p. 21.

THE EFFECT OF DEFICIENT DIETS ON THE TOTAL ASH, CALCIUM AND PHOSPHORUS CONTENT OF BONES [1]

RUTH YEAGER AND JET C. WINTERS
Department of Home Economics, University of Texas, Austin

(Received for publication June 22, 1935)

Winters, Smith and Mendel ('27) published the results of an investigation dealing with the effects of dietary deficiencies on the growth of certain body systems and organs. This study dealt with calorie, protein and mineral deficiencies which were so drastic that growth was practically inhibited; young animals gained only 5 to 10 gm. during a 40-day period. Among others, the observation was made that persistent skeletal growth occurred on all the diets used and was manifested by an increase in body and tail length and an increase in the length of the leg bones and skull. The amount of this skeletal growth, however, varied with the different types of deficiency. The present report describes the total ash, calcium and phosphorus in the bones of animals stunted in the various ways; comparison is made in these respects with normal rats of the same age and with normal rats of the same weight. An attempt is made to relate the mineral content of the bones of the animals stunted in different ways to linear measurement and to weight; and to discover what, if any, differences in the total ash, calcium, and phosphorus content of bone result from the different kinds of dietary deficiency.

[1] From a thesis submitted in partial fulfillment of the requirements for the degree of master of science in home economics, to the faculty of the graduate school, University of Texas, Austin, Texas.

EXPERIMENTAL PROCEDURE

Six groups of rats were used; with one exception each group consisted of twenty-five male animals. There were two control groups, one of which consisted of animals of approximately the same age as the stunted groups (referred to as age controls) and the other of animals of approximately the same weight (referred to as weight controls). The age controls, of which there were only five, were, of course, much heavier and the weight controls much younger than the stunted animals. The figures obtained from the weight control groups were used as a base for estimating percentage increases in weight, length and mineral content of the bones during the stunting period, i.e., it was assumed that the findings for the weight control group were representative of what would have been true for the stunted groups had it been possible to weigh, measure and analyze their bones at the beginning of the stunting period. Allowance for such small increases as might normally occur with a weight increase of 5 gm. (the average increase of the stunted groups) was made by using a weight-control group 5 gm. heavier, on the average, than the stunted group were at the beginning of the experiment. For the stunted groups, animals were started on the experiment at ages varying from 21 to 25 days, but always at a weight of 40 gm. During a 40-day period of stunting no animal gained more than 10 gm., and no group showed an average weight increase of more than 5 gm. The four types of stunting used were 1) low calorie, 2) low protein, 3) low lysine, and 4) low mineral. For the diets and the experimental procedure used to bring about restriction of growth the reader is referred to the report of Winters, Smith and Mendel ('27).

Since all the breeding rats used for the production of the litters involved in the experiment were on the same stock diet, and the rats, after separation from the mother, were kept on the same diet until ready for the experiment, there was little variation in the opportunity for storage of minerals. In distributing litter mates among the experimental and con-

trol groups, care was taken to get as equal a distribution as possible with the large number of animals involved.

The right fore and hind legs of the animals were used for the weight, linear measurement and mineral analysis figures here reported. These bones were prepared by carefully cutting through the joints at the shoulder and thigh and cutting away the outside skin. This preparation was placed in a 2 per cent soap powder solution and kept at a temperature of 95°C., for a period of 1 hour. The skin is then easily pulled away from the bones. The bones were dried at room temperature for a period of 30 days before being weighed and measured.

In order to get samples for analysis, the leg bones of the rats of each group were pooled, folded in cloths, crushed between the jaws of large pliers and then pounded to insure proper breaking. Each of the six composites thus obtained were extracted with hot 95 per cent alcohol for a period of 18 hours and with ether for an equal period of time. They were then freed from ether and dried in an electric oven at 96°C. for 1 hour. Weighed samples of the alcohol-ether extracted bones from each group of rats were ashed by first charring over a low flame and then heating in a gas muffle furnace until constant weight was obtained. This ash was then analyzed for calcium and phosphorus. All determinations were done in triplicate.

The modified method of McCrudden was used to determine the calcium. To determine the phosphorus, the method from the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists ('30) was used.

RESULTS OF EXPERIMENTAL WORK

Table 1 gives the percentage of total ash and of calcium and phosphorus in the alcohol-ether extracted bones of rats held at constant body weight by means of various dietary adjustments, together with similar data for normal rats, 1) of the same age, and 2) of the same weight.

TABLE 1

Percentage of total ash, calcium and phosphorus in bones of animals stunted by dietary deficiency

DIETS	TOTAL ASH	CALCIUM	PHOSPHORUS
Age controls, same age as stunted animals	61.02	23.24	10.95
Weight controls, same weight as stunted animals	51.12	18.20	9.28
Low calorie	58.72	22.02	10.06
Low protein	58.34	21.43	10.31
Loy lysine (gliadin)	57.90	21.50	9.27
Low mineral	45.33	15.65	8.58

DISCUSSION OF RESULTS

Winters, Smith and Mendel ('27) (table 2) showed that the various leg bones of rats stunted in different ways (low calorie, low protein, incomplete protein, and low mineral diets) increased 10 to 23 per cent in length during a period of 40 days. There was little variation with the type of diet used,

TABLE 2

Percentage increase in length of leg bones of rats stunted by deficient diets[1]

	LOW CALORIE	LOW PROTEIN	GLIADIN	LOW SALT
Humerus	13	10	13	10
Ulna	14	13	15	12
Radius	17	18	18	17
Femur	23	18	18	18
Tibia	15	13	15	13

[1] The percentage increases are calculated by comparing the average length of the bones of the animals in the stunted groups with the average length of the bones of normal animals of the same weight.

the leg bones of the low calorie animals tending to be slightly longer and those of the low mineral animals slightly shorter than the others.

The same investigators found (table 3) that the increases in the weight of the leg bones were, except for the low salt rats, much more pronounced than increases in length and that there was considerable variation with the different kinds of stunting.

Inasmuch as increase in weight of bones is due largely to increase in mineral constituents, the large differences between changes in weight and length characteristic of all the deficiencies except that of minerals may be taken to mean that the percentage of ash had been augmented in the bones during the stunting period. An increase in percentage of minerals is characteristic of normally developing bones. The

TABLE 3

Percentage increase in weight of leg bones of rats stunted by deficient diets[1]

	LOW CALORIE	LOW PROTEIN	GLIADIN	LOW SALT
Humerus	74	54	54	5
Ulna and radius	61	51	42	0
Femur	90	65	71	20
Tibia and fibula	63	50	61	14

[1] The percentage increases are calculated by comparing the average weight of the bones of the animals in the stunted group with the average weight of the bones of normal animals of the same weight.

TABLE 4

Percentage increase in total ash, calcium and phosphorus of leg bones of rats stunted by deficient diets[1]

KIND OF STUNTING	TOTAL ASH	CALCIUM	PHOSPHORUS
Low calorie	7.60	3.81	0.78
Low protein	7.22	3.22	1.03
Low lysine (gliadin)	6.78	3.29	0.01
Low salt	— 5.79	— 2.56	— 0.70

[1] The percentage increases are calculated by comparing the percentages of total ash, calcium and phosphorus in the bones of the stunted groups with similar percentages in the bones of normal animals of the same weight.

percentage increases in total ash, calcium and phosphorus are given in table 4 and bear out the idea of increase in the mineral content of bone during the stunting period.

Since the bones of the low mineral animals show such a slight increase in weight in comparison with those of the animals stunted in other ways, and at the same time show an almost equal increase in length, it may be concluded that mineral deficiency does not bring about any greater skeletal

stunting than other deficiencies, but that it does result in bones that are poorer in mineral content. Table 4 shows the decrease in total ash, calcium and phosphorus content of the bones of the animals on the mineral-deficient diet; almost half the decrease is due to loss of calcium. These results may be taken as supplementing those of Sherman and Booher ('31) who found no difference in the growth or physical appearance of rats kept, for varying lengths of time, on diets in which calcium was the sole significant variable. The calcium content of the bodies of such rats, however, varied directly with the calcium intake.

Comparison may also be made of the mineral content of the bones of the stunted animals with that of animals of the same age. From table 1 it can be calculated that the leg bones of rats that were kept on a purified, but supposedly adequate, diet during a period of time equivalent to the stunting period showed an increase of 10 per cent in total ash, 5 per cent in calcium, and 1.68 per cent in phosphorus. In no case do the bones of stunted animals show as great an increase in percentage of total ash, calcium, or phosphorus as do those of these normal animals of the same age. It is evident that the leg bones of animals stunted by means of low calorie, low protein, or incomplete protein diets have a much higher percentage of total ash, calcium and phosphorus than those of the younger animals of the same weight, but a lower percentage than those of the heavier animals of the same age. Stunting by means of a low mineral diet, however, results in loss of calcium, phosphorus, and total ash so that the leg bones of such animals have a much lower percentage of total ash, calcium and phosphorus than those of animals stunted in other ways. It is evident that animals on certain types of deficient diets store calcium and phosphorus even when their general growth is considerably below normal but that the storage is at a subnormal rate. Sherman and McLeod ('25) found that the bodies of rats kept on a diet adequate except for the fat-soluble vitamins contained, at 90 days, a percentage of calcium which was slightly more than was characteristic of normal rats of the same weight but less than half as

great as that characteristic of normal rats of the same age. Similar results were found on a diet deficient in vitamin B, on one deficient in cystine and on one drastically deficient in calories. The same investigators report that animals on a low calcium diet actually lose body calcium.

In bones all of the phosphorus and most of the calcium is stored as calcium phosphate. There are, however, small amounts of other calcium salts, particularly calcium carbonate. The question arises as to whether calcium and phosphorus are always stored in bones in definite ratio or whether the ratio characteristic for animals on a normal diet may be upset by deficient diet. Sherman and Quinn ('26) pointed out that in the bodies of rats on an adequate diet, calcium increased at a more rapid rate than phosphorus. That this holds true for the bones described above is indicated by the finding that the Ca:P ratio in bones of 25-day-old normal rats, on a purified, but supposedly adequate diet, is 1.96, whereas that for rats kept on the same diet until approximately 65 days of age is 2.12. The ratio is increased to 2.30 for rats 125 days of age, according to figures by Brooke, Smith and Smith ('34). The Ca:P ratio in the bones of the animals stunted by low calories and low protein is similar to that of rats of the same age rather than the same weight, being 2.18 and 2.08, respectively. The ratio is somewhat higher for the gliadin stunted animals, i.e., 2.31. This may be accounted for by the lesser supply of phosphorus brought about by substituting a simple protein (gliadin) for a phospho-protein (casein). The Ca:P ratio for the low salt animals is 1.82, showing a larger storage of phosphorus in proportion to calcium than is characteristic even of normal rats of the same weight. Brooke, Smith and Smith ('34) determined the calcium and phosphorus content of the leg bones of two groups of 125-day-old rats; one group had been adequately fed and the other kept on a low salt ration similar to the one used in the present experiment. From their figures Ca:P ratios of 2.30 and 2.07 for the adequately fed and low salt animals, respectively, may be calculated. By determining the amount

of calcium present as carbonate, these investigators showed that the proportion of calcium present in bones as carbonate is less in animals given a low salt diet than in those adequately fed. They interpret the shift in the phosphate to carbonate ratio as being due to the fact that in rats on a low salt ration, calcium is taken from the carbonate of the bones to a greater extent than from the phosphate. The figures obtained in this experiment lend themselves to the same interpretation, but there is also the possibility that a dietetic factor is involved. In the low salt diet a certain amount of phosphorus was supplied by the casein but very little calcium was probably available. Inasmuch as no mineral analysis, except total ash, was made on the diet, no quantitative figures are available. Whether or not the dietetic factor plays any great part could be found by repeating the experiment, using a protein that does not contain phosphorus in the low salt ration. The high Ca : P ratio in the bones of the gliadin-stunted animals lends, as pointed out above, some support to the idea that differences in the amounts of calcium and phosphorus supplied by the diet may affect the Ca : P ratio in the bones.

SUMMARY

The right fore and hind leg bones of groups of young, male, albino rats which had been kept at stationary body weight for a period of 40 days by means of deficient diets (low calorie, low protein, incomplete protein, and low salt) were pooled and analyzed for total ash, calcium and phosphorus. Similar analyses were made on the bones of normal rats of the same weight (weight controls) and on the bones of normal rats of the same age (age controls). The percentages of total ash, calcium and phosphorus in the bones of the stunted rats are then compared with those in the bones of the controls; the results obtained by comparison with the weight controls are taken to represent the amount of the mineral stored during the stunting period.

The bones of animals stunted by means of calorie, protein and lysine deficiency showed a much larger percentage of total ash, calcium and phosphorus than was present in the bones of normal animals of the same weight but a smaller percentage in each case than is found in the bones of normal animals of the same age. Stunting by means of a low salt diet resulted in loss of total ash, calcium and phosphorus so that the percentages in the bones of animals so stunted are considerably smaller than those characteristic of normal animals of the same weight and greatly reduced in comparison with those of normal animals of the same age.

There is some indication that the Ca : P ratio in bones depends, to some extent at least, on the amounts of calcium and phosphorus present in diet.

LITERATURE CITED

BROOKE, R. O., A. H. SMITH AND P. K. SMITH 1934 Inorganic salts in nutrition. VII. Changes in composition of bone of rats on a diet poor in inorganic constituents. J. Biol. Chem., vol. 104, p. 141.

Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, Washington, 3rd ed., 1930.

SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. J. Biol. Chem., vol. 93, p. 93.

SHERMAN, H. C., AND F. L. MCLEOD 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., vol. 64, p. 429.

SHERMAN, H. C., AND E. J. QUINN 1926 The phosphorus content of the body in relation to age, growth and food. J. Biol. Chem., vol. 67, p. 667.

WINTERS, J. C., A. H. SMITH AND L. B. MENDEL 1927 The effects of dietary deficiencies on the growth of certain body systems and organs. Am. J. Physiol., vol. 80, p. 476.

THE AUGMENTATION OF THE TOXICITY OF FLUOROSIS IN THE CHICK BY FEEDING DESICCATED THYROID [1]

PAUL H. PHILLIPS, HONORA ENGLISH AND E. B. HART
Department of Agricultural Chemistry, University of Wisconsin, Madison

ONE FIGURE

(Received for publication, June 10, 1935)

Since fluorine has been shown to inhibit normal respiration in certain tissues of the body by Phillips, Stare and Elvehjem ('34), effort was directed to preliminary experiments to see if the addition of metabolic stimulants would not counteract the influence of NaF feeding. Mild doses of desiccated thyroid and 2,4-dinitrophenol fed at the rate of 5 to 6 mg. daily with 0.07 per cent NaF were without beneficial effect in deterring the appearance and development of fluorine toxicosis during the rapid growing period of the chick (Phillips, '34). On the other hand 0.10 per cent of NaF fed in combination with toxic levels of desiccated thyroid shortened the survival period of the chicks. It appeared that if desiccated thyroid were fed in sufficient quantity to influence the basal metabolism its toxicity was appreciably enhanced by NaF, or vice versa. The present study was undertaken with the view of attempting to determine the influence of NaF poisoning upon animals given desiccated thyroid in doses sufficient to cause a reaction but which were non-toxic in short periods.

In preliminary experiments the tolerance of growing chicks to desiccated thyroid ingestion was established (Phillips, English and Hart, '35). It was found that 0.5 per cent was

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

definitely toxic within 6 weeks. One-tenth, 0.2, and 0.3 per cent levels were without obvious harm except for a response in blood cholesterol. The effects of NaF upon growth in the chick were found to be a slight retardation at a level of 0.07 per cent while 0.09 and 0.10 per cent levels were sufficient to clearly retard growth within a period of 6 weeks.

EXPERIMENTAL

Two successive experiments were made. Day-old White Leghorn chicks were used in each case. The basal ration no. 351 (Kline, et al., '34) was composed of:

Yellow corn	58 parts
Wheat middlings	25 parts
Casein	12 parts
NaCl	1 part
$CaCO_3$	1 part
Yeast	1 part
$Ca_3(PO_4)_2$	1 part
Cod liver oil	1 part
Dried liver residue	10 parts

NaF and desiccated thyroid were administered singly and in combination according to the following plan.

Experiment 35A

Lot	*Ration*
I	Basal ration only
II	Basal ration + 0.22 per cent NaF (0.1 per cent F)
III	Basal ration only with intraperitoneal injections of NaF
IV	Ration of lot II with 0.2 per cent desiccated thyroid
V	Ration of lot II with 0.4 per cent desiccated thyroid
VI	Ration of lot II with 0.6 per cent desiccated thyroid
VII	Basal ration + 0.4 per cent desiccated thyroid
VIII	Basal ration + 0.6 per cent desiccated thyroid

The results from this experiment suggested that the levels of fluorine and desiccated thyroid were sufficient to enhance their combined toxicity over that of either alone. Milder doses were then used in a second experiment with chicks.

Experiment 35B

Lot	*Ration*
I	Basal ration only
II	Basal ration + 0.2 per cent NaF (0.09 per cent F)
III	Basal ration only with intraperitoneal injections of NaF
IV	Ration of lot II with 0.05 per cent desiccated thyroid
V	Ration of lot II with 0.10 per cent desiccated thyroid
VI	Ration of lot II with 0.20 per cent desiccated thyroid
VII	Basal ration with 0.20 per cent desiccated thyroid

The original plan for intraperitoneal injections of NaF was to inject a 0.9 per cent solution of NaF at a level of one-half of the quantity of fluorine ingested by lot II. This level was acutely toxic and killed the birds within 1 to 2 hours. Replacements were made at once and a graded series of injections started. The levels used were 8, 16, 32 and 64 mg. of F per kilogram of body weight. A group of 6-week-old White Leghorn chicks were likewise injected. Physiological saline injections were made daily in several birds to serve as control animals for the lot. In experiment 35B all injections were made at the rate of 34 to 40 mg. of fluorine per kilogram of body weight.

Feed consumption records, growth and survival data were obtained for each experiment.

RESULTS

A gross examination at post-mortem presented an interesting difference in the color of the musculature and in the gizzards of the birds on this experiment. Those fed the basal ration, basal ration and mild doses of desiccated thyroid were normal light-colored carcasses. The gizzards were well muscled while the cornified inner lining was smooth, tough and contained few ulcer-like lesions. The carcasses of the fluorine-fed birds without exception were dark in color and approached a cyanotic condition. The cornified lining of the gizzard was considerably roughened and contained many ulcerated lesions. It was quite fragile and broke easily when subjected to slight tension. In the case of lot III where the birds were injected with NaF solutions the meat was very dark and cyanotic resembling the appearance of the dark

TABLE 1

Summary of the daily ingestion of fluorine and desiccated thyroid in milligrams per kilogram of body weight by weekly periods

WEEK LOT	1		2		3		4		5		6		7	
	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.
Experiment 35A														
I	....	....	....	....	....	....	....	....	....	....				
II	136.4	....	96.3	....	78.7	....	76.3	....	65.7	....				
III	....	....	....	....	....	....	....	....	....	....				
IV	110.5	221.0	92.4	184.8	86.8	173.6	120.2	240.4	....	....				
V	118.6	474.0	....	....	....	....	....	....	....	....				
VI	117.0	702.0	130.0	780.0	92.0	552.0	....	....	....	....				
VII	....	612.0	....	483.0	....	436.0	....	343.0	....	350.0				
VIII	....	678.0	....	683.0	....	565.0	....	636.0	....	554.0				
Experiment 35B														
I	....	....	....	....	....	....	....	....	....	....	....	....	....	....
II	107.0	....	103.8	....	103.8	....	70.0	....	67.0	....	78.5	....	66.1	....
III	38.0	....	38.0	....	38.0	....	38.0	....	38.0	....	34.0	....	40.0	....
V	113.9	63.1	96.0	53.5	73.0	49.0	65.9	36.6	89.3	50.0	92.1	50.8	101.0	56.0
IV	96.6	106.0	102.2	113.0	98.0	109.0	100.0	111.0	97.6	108.0	90.0	95.9	88.9	98.6
VI	85.0	200.0	102.1	223.0	102.4	228.0	91.7	200.0	85.5	186.0	65.0	189.0	93.9	208.0
VII	....	216.0	....	220.8	....	199.0	....	128.0	....	235.0	....	179.0	....	187.0

meat from game birds. The cornified lining of the gizzards from these birds was, however, quite smooth, tough and showed no sign of the ulcers noted in the lots fed NaF.

A transitory paralysis of slight character appeared in nearly all birds fed 0.1 per cent, or more, of desiccated thyroid in combination with NaF. This condition was noted during the third and fourth weeks of the experiment. It automatically disappeared in the course of a few days. The paralysis was not observed to occur in the lots fed NaF or desiccated thyroid alone.

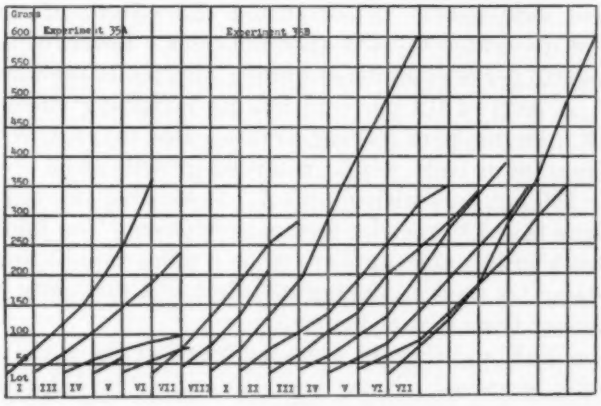


Figure 1

Inspection of table 1 indicates that the ingestion of fluorine ranged from 136 mg. of F per kilogram of body weight during the first week of the experiment to roughly 66 mg. per kilogram of body weight at the close of the experiment. Beyond the third week an average of 66 to 78 mg. of F per kilogram of body weight was ingested. This level of fluorine intake, or more, retarded growth as shown in figure 1. The effect upon growth seems to be a systemic reaction to fluorine. A tolerated level of F compatible with life was apparently reached and food consumption restricted to that level. Normally the feed consumption of growing chicks increases 300 per cent

in 5 weeks and 500 per cent in 7 weeks. The levels of fluorine fed in these studies restricted the increase in feed consumption to 150 per cent at 5 weeks and 300 per cent at the end of 7 weeks. Thus the growth stimulus was held in abeyance by the restriction of feed consumption coincident with the development of fluorosis. That the effect was systemic in nature was further supported by the reaction of lot III to NaF injections. Here too, the feed consumption dropped in the proportions noted when NaF was fed. This evidence indicates clearly that the restriction of feed consumption was not a direct reaction from, or associated with the digestive tract.

These experiments demonstrated the effects of various levels of desiccated thyroid upon the chick. Levels of 0.4 per cent and 0.6 per cent inhibited growth with the latter distinctly toxic. A level of 0.2 per cent had no inhibiting effect upon growth. An intake of 350 mg. of desiccated thyroid, or more, per kilogram of body weight retarded growth while approximately 200 mg. was without effect. Desiccated thyroid when fed alone failed to restrict feed consumption. The inhibition of growth in this case appears to be a more rapid utilization of metabolites thus producing its effect in a distinctly different manner from that of fluorine.

When NaF and thyroid were fed together the toxicity was markedly increased. Every combination of thyroid and NaF in experiment 35A was definitely more toxic than either substance alone. Growth was curtailed sharply. Table 2 indicates that these combinations were sufficiently toxic to produce collapse and death within a very short time. An intake of approximately 200 mg. of desiccated thyroid per kilogram of body weight which in itself was relatively harmless reduced the chick days survived to 60.5 per cent when fed with NaF. This was comparable to the highly toxic level of 0.6 per cent desiccated thyroid fed alone.

In experiment 35B an average daily intake of approximately 50 mg. of desiccated thyroid per kilogram of body weight proved ineffective in accentuating fluorine toxicity. When this level of ingestion was raised to a level of 100 or 200 mg.

per kilogram of body weight the survival period was lowered distinctly. It appeared that approximately 0.1 per cent of desiccated thyroid was necessary to augment the NaF action. At this level of feeding the intake of thyroid and fluorine were practically equal.

The injection of NaF at the rate of 64 or more mg. of F per kilogram of body weight produced rapid collapse characterized by relaxation and extension of the wings, drooped

TABLE 2

Survival data of chicks on experiment

EXPERIMENT 35A				EXPERIMENT 35B			
Lot	Number of chicks	Total number of chick days	Days survived, per cent	Lot	Number of chicks	Total number of chick days	Days survived, per cent
I	6	210	100.0	I	10	490	100.0
II	6	186	88.5	II	10	490	100.0
III	6	...	....	III	10	316	64.5
IV	6	127	60.5	IV	10	490	100.0
V	6	50	23.8	V	10	370	75.5
VI	6	74	35.2	VI	10	337	68.7
VII	6	202	96.2	VII	10	433	88.4
VIII	6	104	49.5				

posture, hyperpnea followed by dyspnea, inactivity, rapid depression and death. Similar symptoms developed after each injection when doses sufficient to retard growth were given. Administration of NaF intraperitoneally was possible up to 40 mg. of F per kilogram of body weight. This level, as well as 16 mg., depressed growth significantly. Eight milligrams of desiccated thyroid per kilogram of body weight were without demonstrable effect upon growth. Similar levels of fluoride injection produced the same type of response in the older chicks.

DISCUSSION

The observations made in this study are of considerable interest in view of the high fluorine tolerance of this species. We have considered that the tolerance of this species might be due to less complete or selective absorption from the digestive tract, a difference in the enzymatic systems involved in cellular metabolism, or to more effective elimination. It is apparent from intraperitoneal injections that the high tolerance to fluorine is not entirely due to incomplete or selective absorption. It appears likely that a combination of the other two factors operate to protect this species. However, the lower storage of fluorine in chronically poisoned hens, reported by Haman, Phillips and Halpin ('35), tends to indicate that more effective elimination plays an important role in the resistance of this species to chronic fluorine toxicosis.

An explanation for the interaction of desiccated thyroid and NaF which results in an augmentation of toxicity is not possible at this time. It seems likely that the results obtained with the chick resemble those with the rat in which the metabolic rate was increased by feeding desiccated thyroid and NaF (Phillips, English and Hart, '35).

CONCLUSIONS

The high tolerance of the growing chick to NaF administration was again demonstrated. It appears that factors other than selective absorption are responsible for the fluorine tolerance of this species. Approximately 70 mg. of F per kilogram of body weight were necessary to inhibit growth in this species after the first week of life. This level of F intake inhibits growth through restriction of feed consumption. The intraperitoneal injection of NaF solutions likewise restricted feed consumption which indicates that the reaction to fluorine was systemic in nature and independent of any reaction via the digestive tract. The tolerance to intraperitoneal injections of NaF solution was distinctly lower, 64 mg. of F per kilogram of body weight were found to be lethal while 35 to 40 mg. per kilogram of body weight retarded growth.

The feeding of desiccated thyroid in amounts greater than 350 mg. per kilogram of body weight definitely retarded growth while levels of 200 to 225 mg. per kilogram of body weight were without effect. Feed consumption was not restricted by desiccated thyroid feeding and differs in this respect from fluorine toxicosis.

Non-toxic levels of desiccated thyroid were made distinctly toxic by chronic fluorosis induced by NaF feeding in the growing chick. Growth was greatly retarded and the survival period shortened. The feeding of the relatively harmless level of 0.2 per cent of desiccated thyroid with NaF produced results similar to that of the highly toxic level of 0.6 per cent desiccated thyroid. Thus fluorine ingestion enhances the toxicity of desiccated thyroid or vice versa.

LITERATURE CITED

HAMAN, K., P. H. PHILLIPS AND J. G. HALPIN 1935 Distribution and storage of fluorine in the tissues of the laying hen. Poultry Science. (In press.)

KLINE, O. L., C. A. ELVEHJEM, J. A. KEENAN AND E. B. HART 1934 Studies on the growth factor in liver. J. Biol. Chem., vol. 107, p. 107.

PHILLIPS, P. H. 1934 Unpublished data, University of Wisconsin.

PHILLIPS, P. H., H. E. ENGLISH AND E. B. HART 1935 Unpublished data, University of Wisconsin.

PHILLIPS, P. H., F. J. STARE AND C. A. ELVEHJEM 1934 A study of tissue respiration and certain reducing substances in chronic fluorosis and scurvy in the guinea pig. J. Biol. Chem., vol. 106, p. 41.

MINIMUM VITAMIN A REQUIREMENTS WITH PARTICULAR REFERENCE TO CATTLE[1]

H. R. GUILBERT AND G. H. HART

Division of Animal Husbandry, College of Agriculture, University of California, Davis

(Received for publication June 3, 1935)

This paper continues the investigation previously reported (Guilbert and Hart, '34). It attempts to elucidate some quantitative vitamin A relationships that are important from practical and from scientific viewpoints. The data presented, although dealing primarily with cattle, so extend the quantitative knowledge concerning vitamin A requirements in widely varying species that some broad generalizations may be made concerning the minimum requirement of mammals.

STORAGE OF VITAMIN A IN CATTLE

In the paper mentioned we showed that in cattle, as in other species, the vitamin A reserve of the newborn is low. The rate of storage, apparently, is relatively slow during the period of rapid growth and increases as the rate of growth decreases, so that maximum storage is obtained in the adult of advanced age. The data were presented in terms of relative values per unit weight of liver. As opportunity afforded, further data have been collected from animals of known history; and from these it has been possible to estimate the total vitamin A reserve in the body.

The carotene determinations were made by the colorimetric method on unsaponfiable fractions of liver and fat tissues,

[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

using the Sprague ('28) dye solutions standardized against beta carotene of high purity obtained through the courtesy of Dr. H. H. Strain, division of plant biology, Carnegie Institution of Washington, Stanford University. Since spectrographic equipment was not available, the vitamin A determinations were made upon the basis of the Carr and Price antimony trichloride reaction, the technic for which has been described (Guilbert and Hart, '34). One color unit by this technic is approximately the equivalent of the color produced by 1 microgram of carotene reacting with 2 cc. of saturated solution of antimony trichloride in chloroform. In the hands of a practiced technician, the variation in judging this end point is about $\pm$ 10 per cent. According to Moore ('33) and others, vitamin A gives approximately ten times as much color with antimony trichloride reagent as does an equivalent amount of carotene. The carotene content and total blue units were determined separately for both liver and adipose tissues. The number of blue units attributable to carotene were deducted from the total blue units; and the remainder, divided by the factor 10, gave a rough estimate of the number of micrograms of vitamin A present. Carotene and total blue-unit determinations were made on both abdominal and back fat, and the mean values were used in the calculations. The total fat in the body was estimated by the formulae of Lusk ('26). Liver weight, when not obtained, was calculated as 1 per cent of body weight. Swett, Graves and Miller ('28) have shown that in very fat beef animals the liver weight may be less than 1 per cent of the live weight, while Swett, Miller, Graves and Mathews ('33) in their extensive data on dairy cattle have found that the liver weight averages about 1.3 per cent of the live weight. Estimating the liver weight, therefore, does not introduce serious errors for the present purpose. In cattle whose livers had 300 to 500 blue units of vitamin A per gram, the lung and spleen extracts gave negative tests for vitamin A, and the kidneys contained only 1 to 5 units per gram. The amount of vitamin A reserve in tissues other than fat, liver and blood serum may be con-

sidered negligible. Semb, Baumann and Steenbock ('34) presented data suggesting that the blood stream of cattle may function as a significant storage reserve for carotene—a source not considered in the data presented in table 1.

Animals nos. 27, 32, 29 and 18 had been used in an experiment on phosphorus deficiency as influencing energy utilization. Nos. 27 and 32 were fed low phosphorus rations, whereas nos. 29 and 18 were the controls. All were placed on experiment at about 1 year of age. Before this time they had access to green feed constantly. During the first 22 months of the experimental period the vitamin A was furnished by 0.9 to 1.4 kg. of fair quality field-cured alfalfa meal and about 30 cc. of cod liver oil daily. During the last 8 months the alfalfa was omitted, and 30 cc. of cod liver oil daily constituted practically the sole source of vitamin A. The feed consumption of these animals was low, especially for those receiving inadequate phosphorus. During the last 8 months the rations contained no roughage. The livers were below average in weight. Although the cod liver oil used was biologically tested by the manufacturer, a later antimony trichloride test showed that it had comparatively low potency. The final reserves of the animals were probably lower than at the beginning of the experiment.

The remainder of the animals were from the university beef herd, and all had had access to green feed and alfalfa hay in abundance throughout their lives. Nos. 26 and 275 had finished lactation periods shortly before being slaughtered. No. 96 had not lactated for about a year. Her liver storage represented the highest values obtained from old cows that have had ample opportunity for storage throughout their lives.

DISCUSSION

According to the data for the first four animals in table 1, the tissues were almost carotenoid-free, and the total reserve was significantly lower than in younger animals having access to abundant green feed. The reserves in the 2-year-old heifers (nos. 469 and 448) were much lower per unit weight of liver

and in total amount than those in the adult cows. The proportion of the total vitamin A reserve found in the body fat varied from 7 to 33 per cent. In the normally fed animals the liver reserve was largely vitamin A, whereas in the fat tissue the blue value obtained only slightly exceeded that attributable to the carotene content, showing that this part of the reserve existed largely as carotene.

TABLE 1

Storage of vitamin A and carotene

ANIMAL NO.	AGE, YEARS	LIVE WEIGHT, KILOGRAMS	LIVER WEIGHT, KILOGRAMS	WEIGHT OF BODY FAT, KILOGRAMS	CAROTENE, MICROGRAMS PER GRAM		VITAMIN A, MICROGRAMS PER GRAM		CAROTENE AND AND VITAMIN A IN MILLIGRAMS		
					Liver	Body fat	Liver	Body fat	Liver	Body fat	Total
27	3½	388	3.05	80[1]	Trace	Trace	31	0.15	95	12	107
32	3½	353	2.77	70[1]	Trace	Trace	35	0.33	97	23	120
29	3½	525	3.27	130[1]	Trace	Trace	42	0.15	137	20	157
18	3½	530	3.60	135[1]	Trace	Trace	50	0.10	180	14	194
469	2	480	4.80[2]	100[3]	3.5	1.7	100	0.60	497	230	727
448	2	523	5.20[2]	105[3]	3.0	1.0	80	0.40	432	147	579
275	6	620	6.20[2]	176[3]	12.0	1.9	200	0.50	1315	422	1737
26	12	566	5.60[2]	85[3]	8.0	6.5	200	0.30	1165	577	1742
96	18	590	5.90[2]	164[3]	6.5	5.2	450	0.30	2693	902	3592

[1] Computed by the formula of Lusk ('26). Percentage of fat in entire animal = 9.073 (per cent of caul fat to live weight) + 0.936 (dressing per cent) — 44.96 per cent.

[2] Estimated as 1 per cent of the body weight.

[3] Estimated by the formula of Lusk ('26). Per cent of fat in entire animal = 1.782 × dressing per cent — 86.4 per cent.

About 225 days were required to deplete the vitamin A reserves of animals whose controls were autopsied and found to have liver storage similar to that of nos. 469 and 448. If it is assumed that the reserves of these animals (nos. 469 and 448) including an average amount in the blood serum, would suffice for a similar period, the indicated withdrawal from storage is 4 to 5 mg. daily, or about 9 to 11 micrograms per kilogram live weight daily.

Because of the limitations in accuracy of the vitamin A determination and the calculations involved, these figures are

admittedly open to criticism; but we present them because they give greater insight into quantitative relationships with regard to cattle than has been obtained heretofore. Baumann, Riising and Steenbock ('34 a) have presented data showing that the rate of depletion of vitamin A stores in rats decreased as the depletion advanced, that is, as the reserves became smaller. Our data on cattle, although limited to a few animals, showed the same tendency. Furthermore, a daily allowance of carotene approximating the minimum requirements for health and normal gain, when added to a carotenoid-deficient basal ration, had little or no sparing action on the rate of loss from storage (Guilbert and Hart, '34). As Baumann, Riising and Steenbock ('34) have shown, the daily intake of vitamin A must considerably exceed the minimum requirement for normal growth before storage occurs. Evidently the intake must be in considerable excess of daily requirement either to maintain already existing reserves or to provide for storage in depleted animals. The estimation of minimum requirement by studying the rate of depletion of reserves, therefore, has limitations. On the other hand the estimate of 9 to 11 micrograms per kilogram live weight appears significant when considered along with the direct determination of carotene requirement of depleted cattle discussed in the next section of this paper.

Young pasture grass or fresh green alfalfa ordinarily contains 35 to 50 mg. of carotene per 100 gm. of dry matter. About 10 kg. of dry matter, therefore, would furnish 3.5 to 5.0 gm. of carotene—an amount equivalent to the estimated maximum storage in a very fat, aged cow that had had access to green feed in abundance throughout her life.

The admission of relatively large errors in the estimations of storage would not detract from the conclusion that cattle ingest in a few days' grazing on green forage more carotene than they store (as carotene and vitamin A) in a lifetime. The fact that during the first 2 or 3 years of life the concentration of reserves in the tissues does not nearly approach that found in aged cattle would indicate that the rate of storage is slow. A depleted cow, no. 440, however, fed about 15

gm. of carotene in freshly cut alfalfa during a 13-day period, stored approximately 400 mg. or 2.7 per cent of that ingested —an average of 30 mg. daily in excess of her daily requirement. The concentration in the liver of 500 blue units per gram was similar to that of 1- to 2-year-old cattle kept under optimum conditions. This fact suggests that storage may be comparatively rapid until a certain level is reached but that further concentration in the tissues may proceed more slowly. The percentage recovery by this cow compares favorably with the recovery of carotene by rats, as reported by Davies and Moore ('34).

MINIMUM CAROTENE REQUIREMENT OF CATTLE

In a study of the effect of various nutritional deficiencies in beef cattle during gestation and lactation, a number of animals were depleted of their vitamin A reserves. During the earlier investigations, animals receiving 1 pound daily of a fairly green chopped alfalfa hay in addition to vitamin A low basal rations showed no clinical symptoms of deficiency after a period 7 months longer than was required to deplete similar animals on the basal ration alone. Two of these animals exhibited no symptoms even though their milk was so deficient in vitamin A that their nursing calves developed definite night blindness and other deficiency symptoms. Thus we had fed empirically an amount of hay with carotene content close to the minimum to prevent the onset of deficiency symptoms. This was the starting point for study of minimum requirement by the curative method with ten experimental animals, nos. 410, 426, 429, 434, 438, 440, G54, 541, 542 and 543.

The concentrate mixtures used in the various basal rations given these animals are shown in table 2.

The carotene content of the concentrate mixtures was less than 0.02 mg. per cent. For roughage, well-bleached wheat or barley straw was used with only a trace of carotene in the extract from 100 gm. Thus the carotene in the basal rations was practically negligible.

The carotene determinations on the alfalfa supplement were made colorimetrically, using the technic described by Guilbert ('35). From November, 1934, the dehydrated alfalfa meal substituted for chopped alfalfa hay was kept in a refrigerator at about 5°C., a temperature which practically eliminates deterioration in carotene content. Samples for analysis were collected as the individual feeds were being weighed into paper bags for each animal. Sufficient feeds were weighed at one time to last 1 to 2 weeks. Carotene determinations on the composite samples were made at monthly intervals. The

TABLE 2

Concentrate mixtures used in various basal rations

FEEDS	CONCENTRATE MIXTURE NO.				
	1	2	3	4	5
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Dried molasses beet pulp	99.0	98.0	84.0	70.0	
Rolled barley				14.0	34.5
Rolled oats					27.0
Wheat bran					27.0
Cottonseed meal			14.5	15.0	10.0
$CaCO_3$	1.0		0.5	1.0	1.5
Mono-calcium phosphate or decarbonized bone black		2.0	1.0		
Per cent digestible crude protein	5.0	5.0	9.5	10.0	12.7
Per cent calcium	1.0	0.9	0.9	0.9	0.7
Per cent phosphorus	0.06	0.4	0.4	0.3	0.7

samples were kept in sealed jars and were stored at a temperature of about —5°C. until analysed.

The nine animals used varied in age from 7 months to 4 years and in weight from 130 to 500 kg. They were fed individually and had the run of paved corrals with access to direct sunlight except at feeding time.

Recovery experiments were started when the animals exhibited complete blindness in semi-darkness. In most cases they also showed nervous symptoms (convulsions). Starting with small amounts of chopped alfalfa hay or dehydrated alfalfa meal of known carotene content, the dose was increased

at intervals until the amount ingested was sufficient to provide for normal weight increases and to cure all clinical symptoms of deficiency. Sub-optimum levels of carotene intake were attained that permitted nearly normal growth yet night blindness persisted. The level of carotene intake at which night blindness disappeared permitted gains up to 1.3 kg. daily. The presence or absence of night blindness, the first detectable clinical symptom of deficiency, therefore constituted a delicate index upon which minimum requirement could be based. All animals were tested for night blindness at frequent intervals by driving them about the corrals in twilight, moonlight, or dim electric light. By this means the normal and deficient animals were readily detected.

The minimum carotene requirement was established by repeatedly increasing or decreasing the intake of alfalfa supplement by each animal until night blindness and other symptoms disappeared or reappeared. The chopped alfalfa hay used varied in carotene content from 2.1 to 3.3 mg. per cent, while the dehydrated alfalfa meal varied from 11.6 to 18.9 mg. per cent. The response in all cases was proportional to the amount of carotene ingested. To obtain further evidence relative to the possibility of factors other than vitamin A being involved, three animals exhibiting deficiency symptoms were given either halibut or cod liver oil. All clinical symptoms disappeared in the same manner as when alfalfa was fed and weight increases were resumed.

After 2 years of experiment with these animals involving long growth periods with the younger animals and the entire gestation period of one of the cows it was evident that the daily minimum requirement of carotene as supplied by alfalfa lay between 26 and 33 micrograms per kilogram live weight. In most instances symptoms recurred when the level of carotene intake fell below 29 micrograms per kilogram live weight. Symptoms regularly reappeared within 2 weeks after the supplement was withdrawn from the ration of animals that had been receiving the minimum level, showing that very little storage occurred.

Three animals were finally allowed to reach advanced stages of deficiency involving total blindness, frequent convulsions, diarrhea, failure of appetite, emaciation and in two of the cases clouding of the corneas. One of the animals died during a convulsion and a histological examination was made of some of the tissues. The remaining two animals were given vitamin A supplements. One of these, a bull, was given two subcutaneous injections of 100 mg. each of carotene dissolved in olive oil, at intervals of 2 weeks. A corneal lesion disappeared in 5 days and slight improvement in physical condition occurred, but there was no weight increase during a period of 1 month. At the end of this time 0.5 cc. of halibut liver oil was administered daily, and was followed by remarkable response both in weight increase and improvement in physical appearance. After 30 days alfalfa was substituted for the liver oil and improvement continued. The animal remained permanently blind. It fully recovered in other respects, was an active breeder and the semen contained motile sperm. The other animal, a heifer, recovered nearly normal vision in daylight, but still shows after several months, defective vision in twilight, regardless of the amount of vitamin A or carotene fed, indicating an impairment of a permanent nature as contrasted with simple night blindness.

DISCUSSION

Minimum requirement. From the above data the daily minimum carotene requirement of cattle is 26 to 33 micrograms per kilogram live weight. Carotene intake at the level of about 29 micrograms per kilogram live weight prevented or cured clinical symptoms of deficiency and permitted normal weight increases when the basal ration was otherwise adequate and not restricted in amount. When the carotene intake fell below this level, clinical symptoms reappeared simultaneously with cessation or reduction in weight increases. No detectable difference in response occurred when the same level of carotene intake was attained by feeding either a field-cured hay or about one-tenth this quantity of dehydrated meal. Cod liver

oil or halibut liver oil produced the same response in deficient animals as alfalfa.

The minimum daily requirement of 26 to 33 micrograms of carotene per kilogram live weight, when furnished by alfalfa, is significantly close to the estimated daily withdrawal of reserves from storage of 9 to 11 micrograms per kilogram live weight, considering the amount of decomposition of ingested carotene that is liable to occur in the digestive tract.

Since the minimum level of carotene intake appeared to be the same per unit of body weight in cattle varying from about 130 kg. to 500 kg., the question arises whether or not vitamin A requirement is related to the amount of body substance or to energy requirement. The differences in body size are not sufficient to give convincing evidence on this point from the cattle data alone. In order to examine this question further, data on species varying in size from the rat to the cow have been assembled in table 3. Comparable data were used with reference to minimum requirement for growth approaching normal and the prevention of clinical symptoms.

When the data are calculated to micrograms of carotene or vitamin A per kilogram body weight, the agreement is exceedingly good in mammals varying in size from 100-gm. rats to 500-kg. cows. Dunlop's data ('34) on swine have rather wide limits: the low level sufficed for nearly normal gains, but deficiency symptoms persisted; the high level exceeded minimum requirement and provided for considerable storage. The minimum requirement, therefore, was probably closer to 13 micrograms per kilogram than to 60 micrograms per kilogram. The mean is, however, fairly close to the values for the rat and the cow.

If the metabolism of rats and cattle were proportional to the body surfaces or the two-thirds power of the body weight then the metabolism per unit of body weight should be 17.1 times greater in 100-gm. rats than in 500 kg. cows. If the metabolism is proportional to the three-fourths power of the body weight (Kleiber, '32) the rat should have a metabolic rate 8.4 times that of the cow. To be proportional to energy

TABLE 3

Daily vitamin A and carotene requirements per unit of body weight of different species

SPECIES	SOURCE OF DATA	APPROXIMATE WEIGHT OF ANIMALS, KILOGRAMS	DAILY INTAKE, MICROGRAMS		GAIN	CAROTENE OR VITAMIN A INTAKE DAILY PER KILOGRAM OF BODY WEIGHT, MICROGRAMS
			Vitamin A	Carotene		
Rat	Moore ('33)	0.150	3.0	3.0	Good	20.0
Rat	Coward ('30)	0.100	2.4[1]	.....	Good	24.0
Rat	Baumann et al. ('34 b)	0.115		2.8	Good	25.0
Swine	Dunlop ('34)	45.000		600.0[2]	Good	13.0
Swine	Dunlop ('34)	45.000		2660.0[3]	Good	60.0
Cattle	Authors data	130.000–500.000		3400.0–16500.0	Good	26.0–33.0 (from alfalfa)
Chicken	Kline et al. ('32)	0.400		30.0	Good	75.0 (carotene in oil)
Chicken	Guilbert and Hinshaw ('34)	0.680		190.0[3]	Good	280.0 (2% alfalfa meal)
Chicken	Guilbert and Hinshaw ('34)	0.680		380.0[4]	Good	560.0 (4% alfalfa meal)
Turkey	Guilbert and Hinshaw ('34)	1.750		468.0[5]	Moderate	270.0 (4% alfalfa meal)
Turkey	Guilbert and Hinshaw ('34)	2.570		1600.0[6]	Good	620.0 (8% alfalfa meal)

[1] Estimated on the basis that 0.3 γ produces the minimum response (1 unit) and that approximately eight times the minimum produces a gain of about 10 gm. weekly, as shown by the curve of response (Coward, '30).

[2] Dunlop ('34) states: "The vitamin A requirement of swine has been shown to lie between 14 and 62 mg. of carotene per 100 pounds of ration." A daily food intake of 4.3 pounds for 100 pounds body weight (the schedule used by Dunlop) was used in making this calculation. The lower level supported nearly normal growth, but deficiency symptoms persisted; on the high level considerable storage occurred.

[3] The carotene intake was about minimum; the amount of storage was small.

[4] The carotene intake exceeded requirements and provided for considerable storage.

[5] The level of carotene intake from 4 per cent of dehydrated alfalfa meal produced slightly less than normal growth with slight storage in surviving birds. There was some mortality with A avitaminosis symptoms.

[6] The carotene intake provided by 8 per cent dehydrated alfalfa meal was adequate for normal growth and a significant amount of storage.

requirement, therefore, the vitamin A requirement per unit of body weight would have to be from eight to seventeen times greater in the 100-gm. rat than in the 500-kg. cow. Since, however, the requirement per kilogram of body weight is practically the same for species varying so much in size and in other characteristics as the rat, the pig, and the cow, we feel justified in advancing the hypothesis that the vitamin A requirement is proportional to body weight and that in mammals it is in the order of 20 to 30 micrograms per kilogram of body weight. Confirmation of this generalization has been obtained in experiments at this station with swine (Hughes and Guilbert, unpublished data) and with sheep (Miller and Guilbert, unpublished data).

If vitamin A requirement is directly proportional to body weight while energy intake is proportional to the three-fourths (or two-thirds) power of the body weight, then, other factors being equal, the percentage of vitamin A in the ration must be increased as body size increases to compensate for the decrease in food consumption per unit of body weight. Some of the errors and difficulties encountered in the past in applying data from one species to another may be explained on this basis. The general impression that young, rapidly growing animals have a higher vitamin A requirement than adults is explained largely by the fact that young animals are depleted sooner than older ones on the same deficient diet. This can be explained on the basis of lower reserves in the young animal and 'dilution' of the reserve as body size increases.

As shown in table 3, the minimum requirement of chickens and turkeys per kilogram of body weight appears to be much higher than that of the mammals studied. In this connection the data of Kline, Schultz and Hart ('32) are especially valuable. Birds receiving daily 30 micrograms of carotene (dissolved in cottonseed oil) gained normally until they attained 400 gm. in weight, then the allowance became inadequate. The experiments of Guilbert and Hinshaw ('34) offer further evidence that the requirement is related to body weight rather

than to total food consumption. Growth, liver storage data and clinical observation all showed that adding 2 and 4 per cent of dehydrated alfalfa meal to an otherwise vitamin A deficient ration fed to chickens gave results comparable to those obtained by adding 4 and 8 per cent, respectively to the same basal ration fed to turkeys. The food consumption of the chickens per unit of body weight, however, was approximately double that of the turkeys, so that the carotene intake per kilogram of body weight was in good agreement, as shown in table 3. The carotene intake by chickens on the 2 per cent alfalfa meal level was slightly more per unit of body weight than that of the turkeys on the 4 per cent level. Symptoms of deficiency developed in some of the turkeys but not in the chickens. This level in chickens appeared close to the minimum and indicates that the availability of carotene in alfalfa for this species is not so high as the availability of carotene in oil.

Requirement for reproduction. The evidence on the minimum requirement for reproduction may be summarized as follows: Five females began their gestation periods with normal storage and were limited to a daily allowance of 9.5 to 15 mg. of carotene, furnished by 1 to 1.5 pounds of alfalfa hay. As has been shown previously, this amount does not prevent storage losses from occurring at about the same rate as when none is fed, at least during the first half of the depletion period. Six calves were produced by these animals. Two successive calves from no. 438 developed diarrhea and died during the first week. Two calves from nos. 410 and G 54, respectively, developed diarrhea, became very weak, but recovered after cod liver oil therapy; one calf from no. 440 recovered from severe diarrhea during the first week without treatment; and one calf from no. 434 was practically normal. The liver of one calf that died contained a trace of vitamin A; the liver of the other contained none. Evidently the mothers' reserves were low, and a carotene intake at approximately the minimum level for the mother was insufficient for the production of normal calves. In contrast to these animals, heifer

no. 426 began her gestation period with no reserve of vitamin A. The daily intake of carotene for the first 8 months of pregnancy (during which she was not lactating) averaged about 30 micrograms per kilogram live weight. During the ninth month the amount was increased to a little more than three times the minimum (90 micrograms per kilogram live weight), and a normal calf was produced. It showed no symptoms of deficiency the first 2 months of nursing, during which the daily carotene intake of the mother was at the level of 143 micrograms per kilogram live weight.

The minimum carotene requirement for the production of milk containing optimal amounts of vitamin A and carotene according to the data of Semb, Baumann and Steenbock ('34) greatly exceeds the animals' minimum requirement plus the amount recovered in the milk.

Nos. 426 and 410 came into oestrum during the time they exhibited night blindness and convusions. No. 410 accepted service but did not conceive.

Clinical symptoms. Night blindness precedes nervous symptoms by one to several weeks. In the early stages, convulsive seizures were sudden, the animal falling as though stunned. Later the animals appeared to feel the attack coming on and braced themselves against falling, swaying from side to side before going down. The attacks lasted from one to several minutes. Total blindness was commonly the next stage and involved, presumably, degenerative changes in the optic nerves. The pupils became widely dilated. At this time a rather characteristic greenish color from the retina is manifested when looking through the cornea with the animal facing the light. Ophthalmoscopic examination made at night, showed this change more distinctly when compared with the yellowish color of the retina in normal animals. Clouding of the cornea appeared, in our experience, in the later stages of deficiency. The onset of symptoms may vary somewhat in different individuals and various localized paralyses may occur.

Blindness at birth in the second calf from cow no. 429 is interesting in the light of previous publications describing

similar conditions. This cow was on minimum vitamin A during her first gestation period, the intake being limited to that contained in 1 pound of alfalfa hay daily from November 23, 1932 to September 26, 1933. On the latter date she was bred, and the alfalfa hay was removed from her diet. She definitely exhibited night blindness on March 6, 1934, in the fifth month of pregnancy. From this time on, ample vitamin A was supplied to ascertain what affect this recognizable deficiency early in pregnancy might have on the offspring.

Crocker ('19) reported blindness caused in newborn calves from what he termed insidious rachitis. There were sixteen cases in Guernseys on two farms in Pennsylvania. No external change was present in the eyes. The dams were all on high concentrate and low roughage feeding for intensive production. On post-mortem the affected calves showed stenosis of the optic canal with chronic optic neuritis, and the condition was diagnosed as insidious rachitis with deformation of the sphenoid bone.

de Schweinitz ('31) and de Schweinitz and De Long ('34) have published on a similar condition in calves from Guernsey cows under the name of papilledema or choked disc, with the suggestion that the defect may be hereditary. A paper by Moore, Huffman and Duncan ('35) describes twenty-four cases in calves and growing dairy animals, of a type of nutritional blindness which the authors state apparently differs from true vitamin A blindness. In their cases blindness without inflammation of the external eye structure was associated with weakness, spasms, and paralysis. We have considered this syndrome in our experimental animals to be characteristic of vitamin A deficiency in cattle, although we have seen marked ulceration of the cornea in some naturally occurring cases (Hart and Guilbert, '33) and in some of the animals in the experiments of Mead and Regan ('31).

Degeneration of nerve tissue, including that of the optic nerve, is well established in vitamin A deficiency. All the cases reported by the workers mentioned above appear to us to be manifestations of vitamin A deficiency. Since the defective sight can be corrected if vitamin A therapy is started

in time, evidently the stenosis of the optic canal is secondary to degenerative changes and to atrophy of the optic nerve.

One case of edema of the legs similar to those reported by Bechdel, Honeywell and Dutcher ('28) was observed. It is suggested that the edema may be concerned with renal involvement. Direct proof, however, is lacking, for the total non-protein nitrogen of the blood serum was normal in our case. No urine examination was made.

Degenerative changes in the kidneys of cattle appear to be common in advanced deficiency. No sperm were found in the testes of bull no. 541 that died of vitamin A deficiency. Although the microscopic picture of sections of testicle and kidney may have been complicated by post-mortem changes, there was good evidence of degenerative alterations attributable to deficiency.

SUMMARY

The total storage of vitamin A and carotene in the liver and body fat of cows 2 to 18 years old, which had access to green feed in abundance throughout life, was estimated to be about 0.6 to 0.7 gm. in the younger animals and up to 3.6 gm. in aged cows. From 67 to 93 per cent of the storage was in the liver. The amount in other organs was negligible. In the liver most of the storage was in the form of vitamin A, whereas in the fat, carotene predominated.

A comparison between the estimated total storage, including that in the blood stream, and the time required to deplete similar animals on a carotenoid-deficient ration, indicated a daily withdrawal of 9 to 11 micrograms per kilogram live weight.

Cattle may ingest more carotene in a few days' grazing on green pasture than is stored in the body of a very fat old cow that has had continual access to green feed or alfalfa hay. A cow whose reserves were depleted stored about 400 mg. of carotene in a 13-day period, during which she ingested 240 kg. of freshly cut alfalfa containing about 15 gm. of carotene. Although the percentage recovery was low (2.7

per cent), a relatively rapid storage was shown. The concentration of the reserves of young animals as compared with that of aged adults, however, suggests that reserves above a certain level are accumulated slowly.

Subcutaneous injections of carotene, dissolved in olive oil, into a vitamin A deficient calf were followed by disappearance of a corneal lesion and by slight improvement in physical condition; but gain in weight did not occur. Apparently the injected carotene was slowly absorbed or poorly utilized.

Night blindness was found to be the first detectable clinical symptom of vitamin A deficiency and to constitute a delicate index upon which minimum requirements could be based. The progressive development of other symptoms of deficiency is presented in the discussion.

The daily minimum carotene requirement of the bovine is 26 to 33 micrograms per kilogram live weight. Carotene intake at the level of 29 micrograms per kilogram daily prevented or cured clinical symptoms, including night blindness, and promoted normal weight increases; yet it resulted in no storage. When the intake fell below this level, night blindness reappeared, and gains decreased.

Normal reproduction occurred in one instance in a cow that began gestation with no reserve and whose carotene intake was maintained at about the minimum level until the last month of pregnancy, when it was increased about threefold.

Vitamin A deficiency that had progressed to the point of night blindness and convulsions did not inhibit the occurrence of oestrus.

From a study of the minimum requirement to prevent or cure symptoms of deficiency and to permit growth at approximately the normal rate in rats, swine, sheep, and cattle, the hypothesis is advanced that vitamin A requirement is related to body weight rather than to energy requirement and that the minimum requirement of mammals is in the order of 20 to 30 micrograms of vitamin A or carotene daily per kilogram of body weight. This implies that, to compensate for lower food consumption per unit of weight, large animals require

a higher percentage of vitamin A in the diet than do small animals.

The carotene requirement per unit of body weight of chickens and turkeys appears to be equal, but the requirement of both species is considerably higher than that of the mammals studied, thus indicating a difference between birds and mammals in this regard.

LITERATURE CITED

BAUMANN, C. A., B. M. RISING AND H. STEENBOCK 1934 a Fat soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, p. 705.

———— 1934 b Fat soluble vitamin. XL. The growth method of determining vitamin A. Ztschr. f. Vitaminforsch., vol. 3, p. 81.

BECHDEL, S. I., H. E. HONEYWELL AND R. A. DUTCHER 1928 The effect of feeding heifers a ration deficient in vitamin A. Penn. Agr. Exp. Sta. Bul. 230, pp. 20–21.

COWARD, K. H., K. M. KEY, F. J. DYER AND B. G. E. MORGAN 1930 The determination of vitamin A. Biochem. J., vol. 24, p. 1952.

CROCKER, W. J. 1919 Blindness in calves due to insidious rachitis. Cornell Vet., vol. 19, p. 171.

DAVIES, A. W., AND T. MOORE 1934 The distribution of vitamin A in the organs of the normal and hypervitaminotic rat. Biochem. J., vol. 28, p. 288.

DE SCHWEINITZ, G. E. 1931 Blindness and papilledema in Guernsey calves. Trans. Am. Ophthal. Soc. 67th Annual Meeting, pp. 1–21.

DE SCHWEINITZ, G. E., AND P. DE LONG 1934 Blindness and papilledema in Guernsey calves. Arch. of Ophthal., vol. 11, p. 194.

DUNLOP, G. 1934 Paralysis and avitaminosis A in swine. J. Agr. Sci., vol. 24, p. 435.

GUILBERT, H. R. 1935 Determination of carotene as a means of estimating the vitamin A value of forage. Anal. Ed. Ind. and Eng. Chem., vol. 6, p. 452.

GUILBERT, H. R., AND G. H. HART 1934 Storage of vitamin A in cattle. J. Nutrition, vol. 8, p. 25.

GUILBERT, H. R., AND W. R. HINSHAW 1934 Vitamin A storage in the livers of turkeys and chickens. J. Nutrition, vol. 8, p. 45.

HART, G. H., AND H. R. GUILBERT 1933 Vitamin A deficiency as related to reproduction in range cattle. Calif. Agr. Exp. Sta. Bull. 560, pp. 1–30.

KLEIBER, M. 1932 Body size and metabolism. Hilgardia, vol. 6, p. 315.

KLINE, O. L., M. O. SCHULTZE AND E. B. HART 1932 Carotene and xanthophyll as sources of vitamin A for the growing chick. J. Biol. Chem., vol. 97, p. 83.

LUSK, J. L. 1926 Practical methods of estimating the proportions of fat and bone in cattle slaughtered in commercial packing plants. J. Agr. Res., vol. 32, p. 727.

MEAD, S. W., AND W. R. REGAN 1931 Deficiencies in rations devoid of roughage for calves. J. Dairy Sci., vol. 14, p. 283.

MOORE, T. 1933 Vitamin A and carotene X. The relative minimum doses of vitamin A and carotene. Biochem. J., vol. 27, p. 898.

MOORE, L. A., C. F. HUFFMAN AND C. W. DUNCAN 1935 Blindness in cattle associated with a constriction of the optic nerve and probably of nutritional origin. J. Nutrition, vol. 9, p. 533.

SEMB, J., C. A. BAUMANN AND H. STEENBOCK 1934 Fat soluble vitamins. XLI. The carotene and vitamin A content of colostrum. J. Biol. Chem., vol. 107, p. 697.

SPRAGUE, H. B. 1928 A convenient method of measuring quantities of chloroplast pigments. Science, vol. 67, p. 167.

SWETT, W. W., R. R. GRAVES AND F. W. MILLER 1928 Comparison of conformation, anatomy and skeletal structure of a highly specialized dairy cow and a highly specialized beef cow. J. Agr. Res., vol. 37, p. 685.

SWETT, W. W., F. W. MILLER, R. R. GRAVES AND C. A. MATHEWS 1933 Variations recorded in the study of the conformation and anatomy of 318 dairy cows having records of production. U. S. D. A. Bureau Dairy Ind. Mim. 589.

IS THE WORK OF THE KIDNEY, DUE TO THE EXCRETION OF UREA, A FACTOR IN SPECIFIC DYNAMIC ACTION?

A. G. EATON, SHIRLEY C. CORDILL AND J. L. GOUAUX
WITH THE TECHNICAL ASSISTANCE OF VERA CLAY
Department of Physiology, Louisiana State University Medical Center, New Orleans

(Received for publication June 12, 1935)

That the kidney performs work in the concentration of the urine cannot be denied. This has been well proved by the experiments of Barcroft and Brodie ('05, '06) and by Hayman and Schmidt ('27–'28) by means of studies of the blood gases and by Tangl ('11, '13) who studied the energy metabolism of dogs before and after complete nephrectomy. Tangl concluded that the energy production of the kidneys was equal to about 8 per cent of the total metabolism. In this paper, we are concerned chiefly with the work of the kidney as a possible cause of the specific dynamic action of proteins.

Tangl ('11) administered 10 and 12 gm. of urea to dogs by stomach and obtained only a slight rise in metabolism. However, in one dog, the intravenous injection of urea caused a considerable increase in oxygen consumption (27 per cent). Lusk ('12) concluded from two experiments on a dog that the administration of urea by stomach tube caused no increase in heat production. These results were confirmed by Grafe ('16). Lublin ('28) reinvestigated the subject and reported an increase in metabolism not only when he administered urea, but also when he secured diuresis by giving large amounts of water by mouth. In his experiments the increased heat production was usually coincident with the greatest diuresis instead of occurring during concentration of the urine.

Borsook and Winegarden ('31 a, b, c) after a mathematical study of many of the important papers concerning the specific dynamic action of proteins came to the conclusion that 25 to 60 per cent of the specific dynamic action of proteins is due to the work of the kidney, that the energy consumed by the kidney is 6 to 11 Cal. per gram of nitrogen excreted and that the efficiency of the kidney is probably not more than 1 to 2 per cent. They also report on experiments carried out using students as subjects. The subjects were apparently untrained and a clinical type of apparatus was used for the metabolic determinations.

METHODS

The three authors and a departmental technician served as subjects for this series of experiments. None of them showed any kidney disease. All were thoroughly accustomed to metabolic determinations, having served as subjects for previous experiments. A light dinner was eaten the night previous to the test, a good sleep of 8 hours or more was obtained and no food or beverage, except water, was consumed until the completion of the experiment. Most of the experimental period was spent resting quietly on the metabolism cot.

One basal determination was made each morning after which the urea solution was drunk. In most cases 30 gm. of urea were given and any exceptions are noted in the table. Four further metabolic determinations were made during the successive 4 hours. Control days, when no urea was taken, were interposed.

The air samples were collected in a Tissot spirometer and analyses of this expired air were made and carefully checked by means of a modified Haldane analyzer accurate to 0.005 per cent. All analyses were made by the same individual. The accuracy of the apparatus was checked by frequent analyses of outdoor air.

Urine samples were collected hourly unless otherwise noted in the table. Each sample was measured and the specific gravity estimated by means of a urinometer. Nitrogen determinations were carried out in duplicate by the usual macro Kjeldahl method.

TABLE 1

Subject I, white female

6/19/34

	Cal./hr.	*cc. urine/hr.*	*Sp. gr.*	*Per cent N_2*	*Gm. N_2/hr.*
Basal	54.2	289	1.004	0.081	0.234
10 gm. urea in 400 cc. warm water and 400 cc. water through day					
1st hour	54.4	316	1.004	0.235	0.739
2nd hour	54.3	274[1]	1.005	0.218	0.597
3rd hour	53.8	274[1]	1.005	0.218	0.597
4th hour	54.0	487	1.002	0.059	0.287

Total change — 0.3 Cal.

[1] Collected and analyzed together.

7/10/34

Basal	54.2	300	1.002	0.081	0.243
20 gm. urea in 400 cc. warm water and 400 cc. water through day					
1st hour	54.0	353	1.002	0.207	0.731
2nd hour	51.0	485	1.003	0.206	0.999
3rd hour	51.8	262	1.004	0.361	0.946
4th hour	51.3	590	1.001	0.146	0.861

Total change — 6.7 Cal.

1/9/35

Basal	56.0	175	1.006	0.229	0.401
400 cc. warm water, no urea					
1st hour	56.4	378	1.001	0.168	0.635
2nd hour	53.6	162	1.005	0.224	0.363
3rd hour	54.6	198	1.001	0.253	0.501
4th hour	52.1	48	1.011	0.476	0.228

Total change — 7.7 Cal.

1/14/35

Basal	52.7	289	1.005	0.143	0.413
400 cc. warm water and 400 cc. warm water through day, no urea					
1st hour	53.7	143	1.005	0.188	0.269
2nd hour	52.9	147	1.003	0.165	0.243
3rd hour	52.7	309	1.002	0.121	0.374
4th hour	55.4	105	1.004	0.269	0.282

Total change + 3.9 Cal.

1/18/35

Basal	54.2	366	1.003	0.162	0.593
30 gm. urea and approximately 600 cc. warm water					
1st hour	53.7	324	....	0.540	1.750
2nd hour	53.8	166	1.013	1.123	1.864
3rd hour	53.4	116	1.015	0.930	1.079
Approximately 500 cc. warm water					
4th hour	54.9	227	1.004	0.437	0.992

Total change — 1.0 Cal.

TABLE 1—*Continued*

Subject II, white female

1/23/35

	Cal./hr.	*cc. urine/hr.*	*Sp. gr.*	*Per cent N_2*	*Gm. N_2/hr.*
Basal	48.7	22	1.026	1.260	0.277
Approximately 600 cc. water and approximately 400 cc. water through day, no urea					
1st hour	53.9	90	1.009	0.538	0.484
2nd hour	52.6	47	....	0.694	0.326
3rd hour	53.1	29	....	0.980	0.284
4th hour	52.4	229	1.004	0.227	0.520
Total change + 17.2 Cal.					

1/28/35

Basal	51.4	21	....	1.565	0.329
30 gm. urea with approximately 1000 cc. water					
1st hour	50.9	200	1.012	0.787	1.574
2nd hour	55.6	164	1.013	1.140	2.312
3rd hour	49.5	112	1.014	1.378	1.543
4th hour	49.5	107	1.017	1.442	1.543
Total change — 0.5 Cal.					

2/6/35

Basal	51.7	61	1.008	0.336	0.205
30 gm. urea with approximately 600 cc. water					
1st hour	49.4	440	1.003	0.319	1.404
2nd hour	52.8	227	1.010	0.675	1.532
3rd hour	51.7	170	1.013	0.973	1.654
4th hour	49.8	160	1.013	1.002	1.603
Total change — 3.1 Cal.					

2/13/35

Basal	51.7	275	1.002	0.085	0.234
30 gm. urea with approximately 600 cc. water					
1st hour	51.1	350	1.004	0.332	1.162
2nd hour	52.0	221	1.013	0.973	2.150
3rd hour	48.9	204	1.011	1.025	2.091
4th hour	54.3	384	1.008	0.449	1.724
Total change — 0.5 Cal.					

Subject III, white male

6/21/34

Basal	65.1	144	1.004	0.224	0.323
20 gm. urea in approximately 500 cc. water					
1st hour	67.3	512	1.003	0.218	1.116
2nd hour	64.5	485	1.003	0.280	1.358
3rd hour	64.5	416	1.002	0.246	1.023
4th hour	64.0	423	....	0.246	1.041
Total change — 0.1 Cal.					

6/29/35

Basal	61.2	139	1.006	0.370	0.514
20 gm. urea in approximately 800 cc. water					
1st hour	63.7	372	1.003	0.302	1.123
2nd hour	63.6	108	1.004	0.633	1.139
3rd hour	60.9	225	1.005	0.546	1.229
4th hour	60.1	300	1.003	0.339	1.017
Total change + 3.5 Cal.					

TABLE 1—*Continued*

	Cal./hr.	*cc. urine/hr.*	*Sp. gr.*	*Per cent N_2*	*Gm. N_2/hr.*
		1/15/35			
Basal	65.4	190	1.007	0.342	0.650
		Approximately 600 cc. water, no urea			
1st hour	61.5	364	1.005	0.207	0.753
2nd hour	66.2	428	1.004	0.176	0.753
3rd hour	66.0	410	1.004	0.221	0.906
4th hour	65.3	258	1.006	0.277	0.715
Total change — 2.6 Cal.					
		1/24/35			
Basal	62.8	120	1.010	0.580	0.696
		30 gm. urea in approximately 500 cc. water			
1st hour	63.9	215	1.007	0.636	1.367
2nd hour	64.2	111	1.013	1.422	1.578
3rd hour	61.8	144	1.010	1.162	1.673
4th hour	62.3	230	1.005	0.613	1.410
Total change + 1.0 Cal.					
		Subject IV, white male			
		7/19/34			
Basal	62.6	85	1.007	0.409	0.348
		20 gm. urea in 400 cc. warm water			
1st hour	61.2	283	1.005	0.314	0.889
2nd hour	63.2	72	1.015	1.033	0.744
3rd hour	62.4	48	1.021	1.413	0.678
4th hour	61.8	37	1.023	1.570	0.581
Total change — 1.0 Cal.					
		7/30/34			
Basal	58.6	187	1.006	0.258	0.483
		30 gm. urea in 400 cc. warm water			
1st hour	57.2	308	1.005	0.403	1.240
2nd hour	67.4	120	1.014	0.999	1.199
3rd hour	57.8	130	1.012	0.904	1.175
4th hour	63.0	330	1.005	0.294	0.970
Total change + 12.0 Cal.					
		12/14/34			
Basal	60.5	119	1.008	0.168	0.200
		30 gm. urea in 400 cc. warm water			
1st hour	60.9	240	1.008	0.684	1.642
2nd hour	61.9	120	1.014	1.218	1.462
3rd hour	64.5	108	1.016	1.224	1.356
4th hour	62.8	97	1.013	1.184	1.148
Total change + 7.1 Cal.					
		12/21/34			
Basal	60.5	46	1.018	0.921	0.424
		400 cc. warm water no urea			
1st hour	58.7	175	1.007	0.331	0.579
2nd hour	59.4	194	1.004	0.269	0.523
3rd hour	62.4	105	1.008	0.400	0.420
4th hour	63.2	63	1.012	0.560	0.353
Total change + 1.5 Cal.					

RESULTS

The table is so nearly self explanatory that only a few points must be mentioned. In all of the experiments on subject I, a total increase in heat production is seen only on January 14, 1935 and that amounted to but 3.9 Cal. It will be noted that on this day no urea was ingested. This rise is in contrast to a fall of 7.7 Cal. on January 9, 1935 which was another control day. Both are well within the range of accuracy of the method. All of the experiments on subject I with the exception of that performed on January 18, 1935 show little concentration of urine as indicated by the specific gravity. This last experiment meets all the requirements of Borsook and Winegarden. There is a very great concentration of the urine. Assuming that the basal nitrogen excretion would have remained constant on this day at 0.162 gm. per hour, the excess nitrogen elimination was 2.382 gm. Calculating the expected increase of 6 to 11 Cal. per gram of nitrogen the least net increase in heat production should have been 14.3 Cal. and the maximum 26.2 Cal. This is in contrast to a net decrease of 1.0 Cal.

The only net increase in metabolism shown in the experiments on subject II was on January 23, 1935 when no urea was ingested. It would seem that the basal metabolism on that day was too low. This subject showed the ability to excrete large amounts of nitrogen and yet the metabolism failed to rise.

Subject III showed a net increase in heat production only on June 29, 1934 and this was too slight to be of any significance. There was a fair diuresis on this day in contrast to January 24, 1935 when the urine was markedly concentrated and only a slight rise occurred.

All of the experiments on subject IV showed slight net increase in heat production except that done on July 19, 1934. The greatest rise was on July 30, 1934 and a large part of this was due to the high metabolism during the second experimental hour. In all other respects this hour differed little from the third experimental hour on the same day. The more

frequent fluctuations in the case of this subject can probably be explained as being due to his lack of ability to relax quickly.

DISCUSSION

Our results are in accordance with those of Lusk and Grafe which are quoted above. They are in contrast to those of Lublin, to one experiment of Tangl in which urea was administered intravenously and to those of Borsook and Winegarden. Lublin administered large quantities of water to his subjects and the increases in heat production which he obtained were usually coincident with diuresis. Hence in his study the work of the kidney in the concentration of the urine could not be an important factor. It seems more probable that the explanation of his results lies in osmotic changes in the blood and tissues. The same explanation probably holds for the experiment of Tangl which is mentioned above. That osmotic changes do increase metabolism has been clearly shown by Wilhelmj, Bollman and Mann ('31). The experiments of Borsook and Winegarden are open to criticism since they used untrained subjects and a clinical apparatus not designed for extreme accuracy.

Our experiments rule out the work of the kidney in the concentration of the urine as a factor in the specific dynamic action of proteins. They do not eliminate the kidney as a possible seat for other reactions dealing with the increased heat production following ingestion of proteins or amino acids. Lusk ('31) has concluded that the cause of the specific dynamic action of proteins is probably the various intermediary reactions through which the amino acids pass during their metabolism in the body. We are now attempting to investigate whether or not some of these may occur in the kidney.

CONCLUSION

The work done by the kidney in the concentration of the urine during rapid excretion of urea is not a material factor in the specific dynamic action of proteins.

LITERATURE CITED

BARCROFT, J., AND T. G. BRODIE 1905 The gaseous metabolism of the kidney. J. Physiol., vol. 32, p. 8.

———— 1906 The gaseous metabolism of the kidney. J. Physiol., vol. 33, p. 52.

BORSOOK, H., AND H. M. WINEGARDEN 1931 a The work of the kidney in the production of urine. Proc. Natl. Acad. Sci., vol. 17, p. 3.

———— 1931 b The energy cost of the excretion of urine. Proc. Natl. Acad. Sci., vol. 17, p. 13.

———— 1931 c On the specific dynamic action of protein. Proc. Natl. Acad. Sci., vol. 17, p. 75.

GRAFE, E. 1916 Beiträge zur Kenntnis der Ursachen der spezifischdynamischen Wirkung der Eiweisskörper. Deut. Archiv. klin. Med., Bd. 118, S. 1.

HAYMAN, J. M., JR., AND C. J. SCHMIDT 1927–1928 The gaseous metabolism of the dog's kidney. Am. J. Physiol., vol. 83, p. 502.

LUBLIN, A. 1928 Über den Einfluss der Nierenarbeit auf den Gaswechsel des Menschen. Zeitschr. für klin. Med., Bd. 109, S. 371.

LUSK, G. 1912–1913 Animal calorimetry. III. Metabolism after the ingestion of dextrose and fat, including the behavior of water, urea and sodium chloride solutions. J. Biol. Chem., vol. 13, p. 27.

———— 1931 The specific dynamic action. J. Nutrition, vol. 3, p. 519.

TANGL, F. 1911 Die Arbeit der Nieren und die 'spezifischdynamische Wirkung' der Nährstoffe. Biochem. Zeitschr., Bd. 34, S. 1.

———— 1913 Calorimetrie der Nierenarbeit. Biochem. Zeitschr., Bd. 53, S. 36.

WILHELMJ, C. M., J. L. BOLLMAN AND F. C. MANN 1931 A study of certain factors concerned in the specific dynamic action of amino acids administered intravenously and a comparison with oral administration. Am. J. Physiol., vol. 98, p. 1.

THE EFFECT OF MALE HORMONE ON THE PROTEIN AND ENERGY METABOLISM OF CASTRATE DOGS [1]

CHARLES D. KOCHAKIAN AND JOHN R. MURLIN

Department of Vital Economics, University of Rochester, Rochester, New York

FIVE FIGURES

(Received for publication June 8, 1935)

INTRODUCTION

The relationship of the male gonads to metabolism has been the subject of investigation of many workers. Korenchevsky ('25) in the first of a series of articles on sex glands and metabolism, summarizes the results of castration on energy and protein metabolism obtained by various workers on both animals and humans since 1898. The results of the various investigators vary. Korenchevsky's ('25) own investigations indicated that after castration two types appear, a 'fat' and a 'thin,' in both humans and dogs. In fat castrated dogs energy metabolism and nitrogen metabolism were both decreased after castration. On the other hand in 'thin' castrated dogs this fall in the nitrogen and gaseous metabolism was usually less pronounced or absent; moreover, the gaseous metabolism was sometimes considerably increased.

Koch ('32) reviewing the results obtained by numerous workers after the injection of testis emulsions and extracts was of the opinion that the evidence for a gonadal hormonal influence on metabolism was very unconvincing.

[1] This investigation was aided by a grant from the Committee on Scientific Research of the American Medical Association.

Schrire and Zwarenstein ('32 a) reported that castration of adult male rabbits causes an increase of 25 to 50 per cent in creatinine output. This abnormality could be corrected by the grafting of testis and by the injection of saline suspensions or extracts of testes ('32 b).

Read ('21) found that the metabolism of Pekin eunuchs was greatly deranged like that of a cretin. Shen ('25) on the other hand was unable to find any creatine or other abnormalities in the nitrogen metabolism of eunuchs.

McCullagh, McCullagh and Hicken ('33) in a study of the clinical treatment of hypogonadism with male sex hormone, reported that after treatment there was a rise in basal metabolism and a fall in the level of blood cholesterol.

In view of this conflicting and unconvincing evidence, this study was undertaken. It has already been amply proven that the testes elaborate a hormone or hormones, presumably excreted in the urine, which affect the secondary male characteristics. It was considered advisable, therefore, to attack the problem of a gonadal hormonal relationship by studying first the effect of this male sex hormone or hormones on the metabolism of castrate dogs.

PREPARATION OF THE HORMONE

Since male sex hormone can be readily prepared from urine and that source of supply was conveniently available,[2] the starting material used in this study was urine. The method of Funk and Harrow ('30) was found to be easily adaptable and this method with two modifications was used.

The urine, which was collected in 18-liter bottles with or without preservative, was made acid by the addition of concentrated hydrochloric acid and then an additional 20 cc. of acid per liter of urine was added. Funk and Harrow ('30) found that extraction of urine so treated always resulted in the formation of an emulsion between the solvent and the urine. We were able to successfully overcome the formation

[2] The authors wish to express their appreciation to the medical students for their cooperation.

of this emulsion by concentrating the acidified urine to about one-eighth its volume in large evaporating dishes on an active steam bath. If, however, the urine was not acidified before concentration, or the steam bath was not very active, the emulsion would still be formed. Evidently, therefore, there is a breakdown of the emulsifying agents in the urine under the influence of acid and heat. The concentrated urine was extracted under reflux for 10 to 12 hours with chloroform, the chloroform was separated from the watery layer and the chloroform removed by distillation. The residue was heated under reflux for about 1 hour with 1 gm. of sodium hydroxide dissolved in 5 cc. water for each 3 liters of urine. The alkaline residue was then cooled and transferred to a separatory funnel with several small washings of water and ether. The diluted residue (dilution three to four times) was extracted eight to ten times with ether. If the ether was removed at this point and the residue was dissolved in olive oil, there would be considerable tarry material which would not dissolve in the olive oil. If, however, the ether extract was washed twice with dilute hydrochloric acid, the insoluble material would readily precipitate out. The ether extract was then washed twice with water, the ether removed and the final product, a reddish viscous oil, was completely soluble in olive oil, but on allowing to stand in the refrigerator for 3 to 4 days, more of the above inert material precipitated out.

The method as finally adopted has yielded quantitative results as determined by the extraction of approximately 4000 liters of urine in batches of 1 to 1000 liters.

ASSAY OF THE HORMONE

The hormone was dissolved in olive oil so that 1 cc. of the oil solution was equivalent to 4 liters of urine. The potency of the solution was determined qualitatively by the regeneration of the prostate and seminal vesicles of castrate rats and quantitatively by its effect on the combs of White Leghorn capons, according to the method of McCullagh, McCullagh and Hicken ('33). The hormone solution, 0.05 cc., was injected

into the pectoral muscles for 2 successive days and a maximum growth of length plus height (L + H) was obtained on the fourth day. The results obtained are listed in table 1 and are the same for the different preparations, i.e., 25 to 34 B.U. per cubic centimeter according to the McCullagh technic.

DESCRIPTION

Three dogs were used. Dog 1 was castrated in November, 1931 and has always been thin and very active. Dog 2 was castrated in November, 1933 and soon became fat and very inactive, the direct opposite of dog 1. Dog 3 was castrated[3] in November, 1934. At the time of castration it was approximately 10 months old and had been in confinement for 4 months. Castration to date has not shown any significant effect on its basal metabolism, as calculated by the Cowgill-Drabkin method ('27) for Cal./sq.m./hr. This dog has not shown any tendency to become a fat castrate.

TABLE 1

Assay of the hormone preparations (0.05 cc. injected per day)

PREPARATION NUMBER	DATE	INCREASE OF LENGTH + HEIGHT	AVERAGE INCREASE
II	2/4/35	+ 6, + 4, + 5	+ 5
III	2/4/35	+ 5, + 4, + 6	+ 5
X	12/11/34	+ 8, + 4, + 7	+ 6
	1/7/35	+ 6, + 6	+ 6
	3/27/35	+ 4, + 4	+ 4
	4/17/35	+ 6, + 4	+ 5
67	4/17/35	+ 5, + 5, + 5, + 5 (+ 11)	+ 5

Diets. The dogs were maintained on diets which proved adequate in all respects and at no time did the dogs show any impairment of appetite or other dietary deficiencies.

Dogs 1 and 2 were placed on the diet shown in table 2.

The nitrogen content of the beef heart varied but analyses were made of each beef heart at the time of feeding. The cracker meal was kept in a tightly sealed glass container. All

[3] The authors wish to express their appreciation to Mr. Teufel and Mr. Disbro, medical students, for their assistance in training and castrating this dog.

analyses were made on aliquots by the Kjeldahl-Gunning method.

The diet was prepared by melting the lard to which was added the beef heart, previously cut into small squares, and the other constituents plus about 700 cc. of water.

TABLE 2
Beef heart diet

	DOG 1, WEIGHT 12 KG.			DOG 2, WEIGHT 22 KG.		
	Grams	Grams nitrogen	Calories	Grams	Grams nitrogen	Calories
Beef heart	150	4.50	372	200	6.00	500
Cracker meal	60	1.16	233	85	1.67	332
Lard	20	....	180	24	....	180
Cod liver oil	5	....	45	5	....	45
Bone ash	5	....	....	10	....	....
Wesson's salt mixture	3	....	....	3	....	....
Totals		5.66	830		7.67	1057

TABLE 3
Cowgill's diet

	DOG 3, WEIGHT 20.9 KG.		
	Grams	Grams nitrogen	Calories
Casein	109.3		405.7
Sucrose	102.3		409.2
Bone ash	4.8	13.79	
Wesson's salt mixture	2.4		
Yeast powder [1]	1.2		4.0
Lard	50.0		450.0
Cod liver oil	5.0		45.0
Totals		13.79	1313.9

[1] Standard brand unirradiated.

Dog 3 was placed on the diet prescribed by Cowgill ('23). The first five constituents of the casein diet were prepared in a large batch and placed in a tight tin container. Aliquots of this mixture were analyzed for nitrogen. The diet was prepared by mixing the dry constituents and cod liver oil with the melted lard.

PROCEDURE

The dogs were confined in metabolism cages and fed the prescribed diet at approximately 4 P.M. every day. The dogs were catheterized just before feeding daily or at the end of each period (5 days each) depending on the type of experiment. The urine was collected under toluene daily and saved in the refrigerator until the end of the period when analyses were made. Total nitrogen of the urine was determined by the Kjeldahl-Gunning method, ammonia and urea by the Van Slyke-Cullen ('16) aeration method, creatine and creatinine by the Folin ('14) microchemical method. The rest nitrogen was determined by difference.

The fecal periods were terminated with the use of carmine as a marker. The feces were collected daily and saved in the refrigerator in acidified alcohol. At the end of the period the feces were dried on a steam bath, powdered and aliquots analyzed for nitrogen.

The energy metabolism of dogs 1 and 3 was determined by means of a Benedict closed circuit apparatus, attached to an all metal chamber, which was enclosed by a water jacket. Frequent alcohol checks were made to be certain that the apparatus was air-tight and working properly.

The Tissot-Haldane method with face mask was used for dog 2.

The three dogs were thoroughly trained to their respective regimes. Activity records of dogs 1 and 3 were obtained by means of a kymograph. Dog 2 was kept under observation and respiration rates recorded. Only those experiments were used in which the dogs were quiet and in a basal condition. Dogs were fed regularly at 4 P.M. and all energy metabolism experiments were begun at least 17 hours later. Temperature of the chamber was always between 22° and 27°C. and, except in the case of experiment no. 1 on dog 1, as mentioned on page 447, the animals were kept in the laboratory throughout the experimental period so that no temperature readjustment was in question (aside from the instance given).

RESULTS

Successive injections

The experiments on protein and energy metabolism were carried out simultaneously. At first, however, we were only concerned with the energy metabolism. Therefore, preliminary experiments were made on the effect of large single injections on energy metabolism alone. Since these experiments, which will be discussed later, yielded negative results, continuous injections for a period of time were carried out. Four such experiments were carried out with dog 1 and three with dog 2. A summary of the nitrogen balances of these experiments are presented in table 4.

TABLE 4

Summary of nitrogen balances, grams per period (5 days)

	DOG NO. 1				DOG NO. 2		
Bird units (B.U.) male hormone per day	13–17	25–34	38–51	25–34	25–34	38–51	13–17
Experiment no.	1	2	3	4	1	2	3
Periods before injection							
1	— 0.10	+ 0.30	+ 0.05	+ 0.65	+ 4.75	+ 0.45	
2			+ 0.80	+ 1.30		+ 1.10	+ 0.45
Injection periods							
1	+ 1.45	+ 2.65	+ 2.55	+ 2.40	+ 4.50	+ 2.90	+ 1.70
2	+ 3.05	+ 3.45	+ 2.35	+ 3.10	+ 6.35	+ 6.45	+ 0.85
3	+ 2.85	+ 5.05	+ 0.50	+ 4.25	+ 6.45	+ 6.55	+ 1.65
4			+ 2.45		+ 6.60	+ 6.55	+ 2.60[3]
5			+ 3.65				
Periods after injection							
1	+ 3.15	+ 3.00	+ 1.30	+ 4.20[1]	+ 4.05	+ 3.90	+0.55[3]
2	0.00	+ 0.75	— 1.00	+ 1.90	+ 1.00	— 2.75	— 0.45
3		+ 0.55[1]	+ 0.65	+ 2.10	— 1.75[4]	— 1.50	— 0.35
4			+ 1.30	+ 2.50	— 1.30	— 1.45	0.00
5				+ 0.70	+ 0.40[2]	— 0.65	
6				+ 2.60	— 1.55	— 0.30	
7					0.00	— 1.20	
8					— 0.50	— 1.05	
9					+ 0.45	+ 0.45	
10					+ 1.10		

[1] Four-day period, but calculated for 5 days.

[2] Six-day period, but calculated for 5 days.

[3] Seven-day period, but calculated for 5 days.

[4] Three days lost between this and the preceding period.

In the seven experiments recorded, a single preparation, X, which was assayed repeatedly (table 1, p. 440) during the course of the experiments was used. No toxic effects were noted and the hormone solution was readily absorbed. In every experiment except the last, the hormone was injected subcutaneously in half doses every morning and evening. In the last experiment the hormone was injected in a single dose every morning.

The results for dog 1 indicate that there is an increased nitrogen retention with increased dose up to a certain point (experiment 2), beyond which (experiment 3) there is no greater response and possibly a decrease. Experiment 4 was run immediately after experiment 3 to see if experiment 2 could be duplicated. As figures show, there is fairly good duplication.

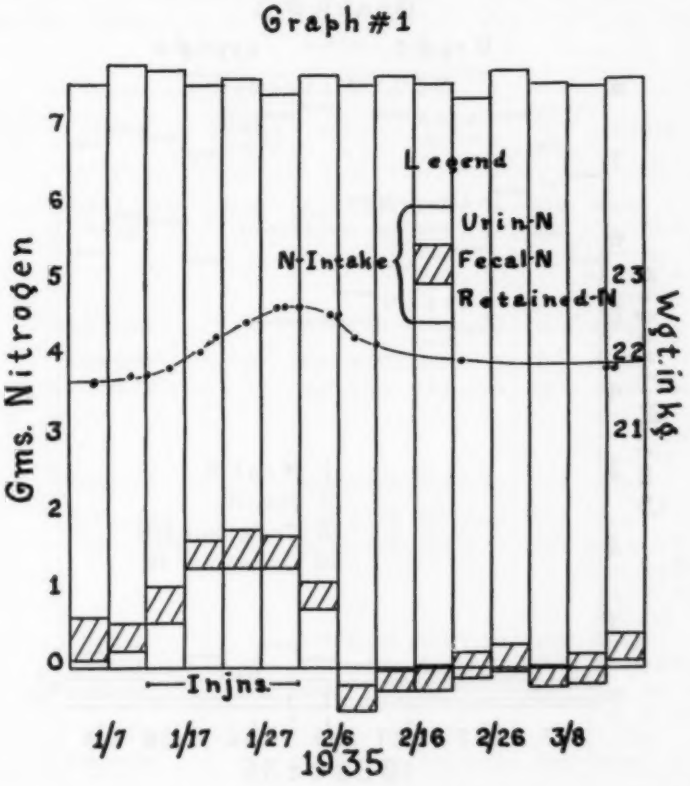
Experiments 1 and 2 of dog 2 yielded remarkably constant and identical results. Undoubtedly, this is due to obtaining a maximum effect with the first dose, beyond which no greater effect resulted. The third experiment, therefore, was designed to determine whether a smaller dose would produce the same effect. As may be seen, table 4, decreasing the dose to half that of experiment 1, produced only about 30 per cent the effect. The high basal value obtained in experiment 1 was due to a personal fault in that sufficient time was not given the dog to come into nitrogen equilibrium. It does not, however, detract from the significance of the results, for comparatively it affected only the first injection period.

In every case dog 2 went into a sustained minus balance shortly after cessation of injections. This minus balance attained a maximum immediately and gradually returned to basal. The excess nitrogen output, however, never attained the proportions of the amount retained. In experiment 3 the amount and duration of the minus balance is correspondingly shorter.

The three experiments were run successively.

The graphical presentation of experiment 2 of dog 2, (graph 1) illustrates clearly the effect of male sex hormone on the

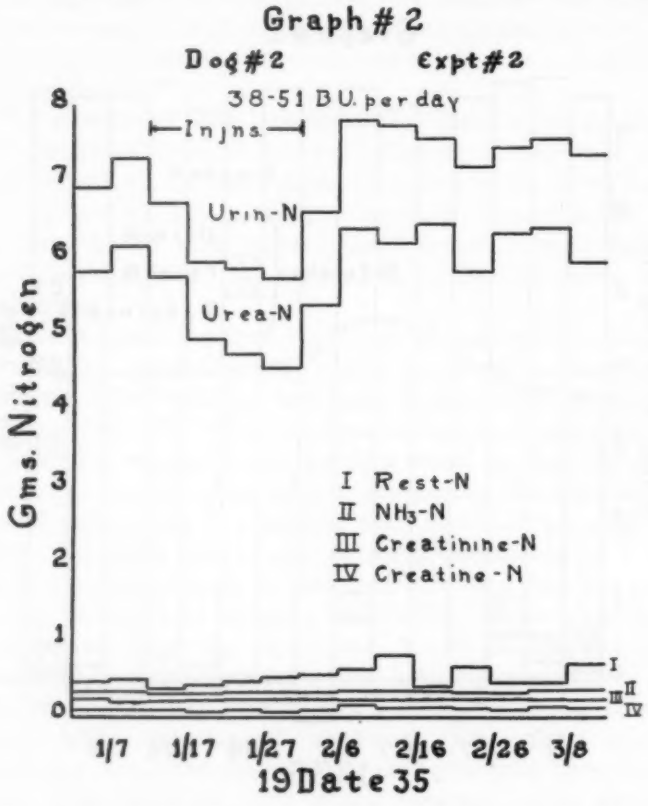
nitrogen balance and also on the dog's weight. The values are represented so that the nitrogen intake is divided into urinary nitrogen, fecal nitrogen and nitrogen retained. The latter, of course, only when the dog is in positive nitrogen



balance. The changes in the nitrogen balance are borne by the urinary nitrogen and never by the fecal nitrogen. The weight of the dog gradually increases and then gradually returns to basal after cessation of injections.

In order to determine what constituents of the urinary nitrogen were affected, partitions of the urinary nitrogen

were made. Graph 2 shows typical results. The significant fact to be noted is that the urea nitrogen parallels the changes in urinary nitrogen. The ammonia and creatinine remain constant. The rest nitrogen shows a variable increase and



creatine nitrogen always increases slightly after cessation of injections.

ENERGY METABOLISM

Although there is a similar effect on the protein metabolism of both dogs, the effect on the total energy output is somewhat different. Tables 5 and 6 summarize the results for

dogs 1 and 2, respectively. The values in table 5, dog 1, are for total R.Q.'s, the values in table 6, dog 2, are calculated for non-protein R.Q.'s according to Lusk ('24, '28). The calories per square meter per hour are calculated according to the surface area formula for dogs proposed by Cowgill and Drabkin ('27).

TABLE 5

Summary, energy metabolism in dog 1

EXPERIMENT NO.	DATE	NUMBER DETERMINATIONS	TOTAL R.Q.	CALS./SQ.M./HR.	WEIGHT AT END OF PERIOD	REMARKS
					kg.	
1	4/15–4/18/34	3	0.74 ± 0.01	30.9 ± 0.4	13.4	Basal
	5/5–5/16	11	0.78 ± 0.02	29.4 ± 0.5	13.5	13–17 B.U. per day (½ cc. X) 5/3–5/18
	5/20–5/25	6	0.79 ± 0.02	28.3 ± 1.6	13.4	Basal
2	6/26–6/27	3	0.77 ± 0.02	28.9 ± 0.7	12.8	Basal
	6/28–7/12	5	0.75 ± 0.01	29.1 ± 1.0	13.1	25–34 B.U. per day (1 cc. X) 6/28–7/12
	7/14–7/27	5	0.75 ± 0.02	28.6 ± 1.1	12.9	Basal
3	12/13–12/20	6	0.77 ± 0.02	29.7 ± 0.9	11.9	Basal
	12/20–1/14–35	8	0.75 ± 0.02	29.4 ± 1.0	12.2	38–51 B.U. per day (1½ cc. X) 12/20–1/14
	1/15–2/1	7	0.76 ± 0.01	28.6 ± 1.2	11.9	Basal
4	2/5–2/15	5	0.75 ± 0.01	29.4 ± 0.9	12.1	25–34 B.U. per day (1 cc. X) 2/3–2/18
	2/20–3/20	5	0.76 ± 0.01	29.2 ± 0.6	12.0	Basal

Dog 1 shows a remarkable constancy in every experiment with the exception of no. 1, which shows a gradual decrease. This, however, is probably not due to the effects of the hormone but to the fact that the dog was acclimating itself to the temperature of the laboratory after its stay in the animal house, which was much cooler.

Dog 2, table 6, on the other hand, shows definite increase in total energy output in two of the three experiments after administration of the hormone. Experiment 3 shows a smaller and rather doubtful increase. It must be emphasized that the increase is never very great. The increase in energy metabolism does not begin immediately, but only after several days;

TABLE 6

Summary, energy metabolism dog 2

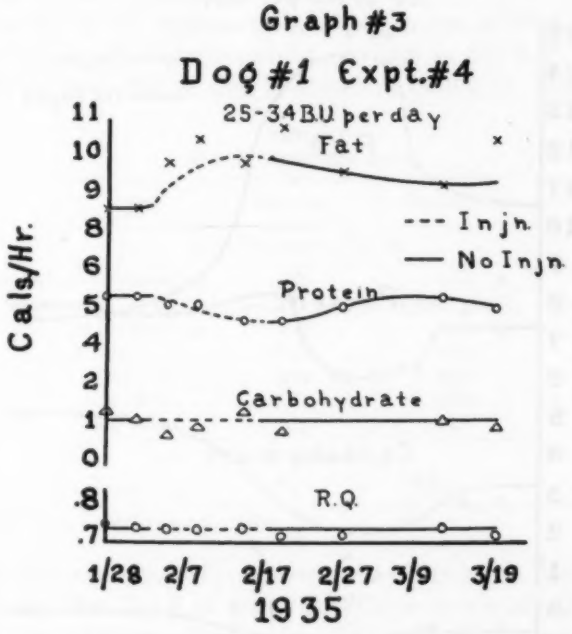
EXPERIMENT NO.	DATE	NUMBER DETERMINATIONS	NON PROTEIN R.Q.	CAL./SQ.M./HR.	WEIGHT END OF PERIOD	REMARKS
					kg.	
	10/31/34					25–34 B.U. per day (1 cc.X)10/31–
1	11/14–11/20	7	0.78 ± 0.01	26.2 ± 0.7	22.2	11/20/34
	11/21–11/26	4	0.79 ± 0.02	25.2 ± 0.7	22.4	Aft. injection
	12/1–1/10/35	11	0.78 ± 0.03	24.1 ± 0.7	21.8	Basal
	1/12–1/15	2	0.74 ± 0.03	24.5 ± 0.1	21.8	38–51 B.U. per day (1½
2						cc. X) 1/12–
	1/19–2/1	17	0.73 ± 0.03	27.1 ± 0.5	22.7	2/1
	2/6–2/16	5	0.77 ± 0.04	26.1 ± 0.2	22.2	Aft. injection
	3/4–3/14	7	0.82 ± 0.02	24.6 ± 0.5	21.9	Basal
	3/21–4/6	13	0.82 ± 0.02	25.4 ± 0.5	22.3	13–17 B.U. per day (1
3						cc. X) 3/18–
						4/16
	4/8–4/15	4	0.83 ± 0.02	25.4 ± 0.0	22.3	Aft. injection
	4/18–4/24	6	0.85 ± 0.02	23.0 ± 0.5	22.3	Basal

later it attains a maximum and continues at that rate for a time after cessation of injections.

In order to determine whether the daily injections had any transient effect, determinations were made on both dogs before the morning injection and at various time intervals after the injections. No effects were detected. Therefore, the increase noted for dog 2 is due to the stimulation of the repeated injections.

In experiment 1 no determinations were made before or during the first part of the injection period. The subsequent results and the results obtained in experiments 2 and 3 substantiate the results obtained in experiment 1.

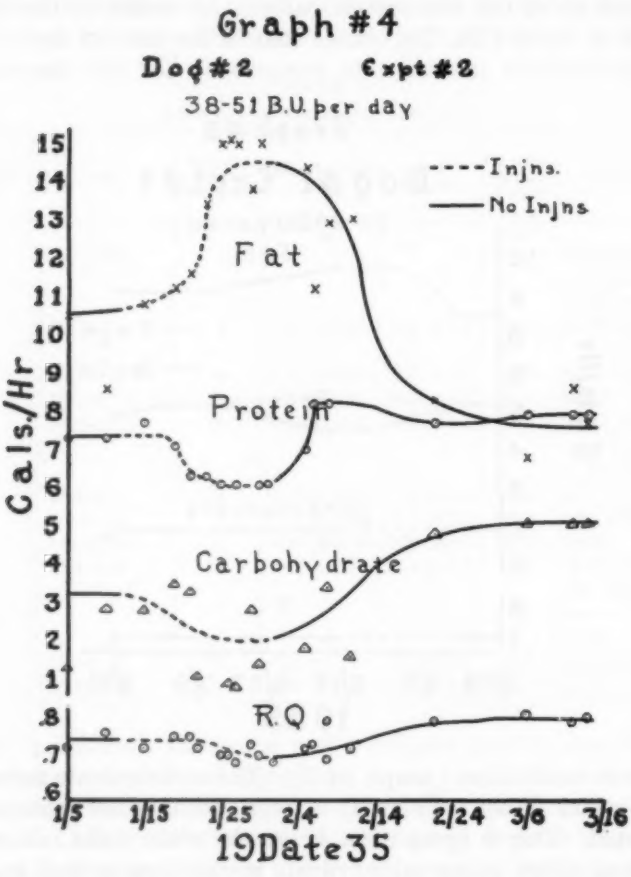
Analysis of the non-protein calories by means of the table given by Lusk ('24, '28) shows that in the case of dog 1 the fat metabolism increases to compensate for the decreased



protein metabolism (graph no. 3). The carbohydrate metabolism which is not very great to begin with, is not materially affected. Dog 2 (graph no. 4) on the other hand, shows a sparing effect in the carbohydrate metabolism as well as the protein metabolism and an increase in fat metabolism to compensate for these sparing effects and also to bear the increased total energy output.

Single large injections

In view of the marked effect obtained on the protein metabolism of both dogs after the continuous injection of male



sex hormone, it seemed advisable to reinvestigate the effect of single injections on the energy metabolism and at the same time observe the protein metabolism.

In the preliminary experiments in which only the energy metabolism was studied, the results in table 7 are typical.

As may be seen, there is no effect as much as 6 hours after the injection. Subsequent experiments in which observations were made as much as 11 hours after the injection and on subsequent days, showed similar results. The doses used were 33 cc. (825 to 1120 B.U.) preparation III, dog 1; 8 cc. (200 to 272 B.U.) preparation II, and 9.1 cc. (208 to 310 B.U.) preparation 67, dog 2; and 5 cc. (125 to 170 B.U.) and 9.4 cc. (235 to 319 B.U.) preparation 67, dog 3.

A study of the protein metabolism in one of these experiments on dog 2, injection of 9.1 cc. (208 to 310 B.U.) preparation 67, and one on dog 3, injection of 9.4 cc. (235 to 319

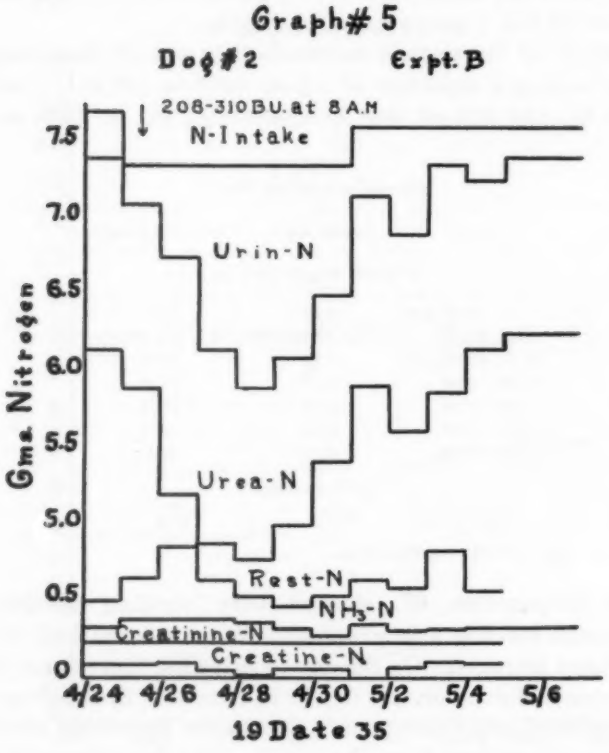
TABLE 7
Energy metabolism dog 1

TIME	TOTAL R.Q.[1]	CALS./SQ.M./HR.
2/22/34 weight 14.2 kg.		
10.05 A.M.	0.73	35.6
11.10	10 cc. preparation II (50 l. urine)	
12.01–12.58 P.M.	0.75	34.8
12.58– 2.00	0.73	37.0
2.00– 3.04	0.74	35.3
3.04– 4.03	0.74	35.0
4.03– 5.00	0.77	35.3
2/27/34 basal		
10.48–11.43	0.74	35.2

[1] EtOH check 3/16/34—0.665, 0.664.

B.U.) preparation 67, showed very striking results, as illustrated for dog 2 in graph 5. There is a marked change in urinary nitrogen. On the day of injection there is no effect. The apparent drop on this day in urinary and urea nitrogen is proportional and consequently due to the decreased nitrogen intake, as is evident from the graph. The day after injection, however, shows a decrease which becomes successively greater and reaches a maximum on the third day and then returns in a similar manner to normal. Partition of the urinary nitrogen shows the urea nitrogen paralleling the changes in the total urinary nitrogen, while the creatinine nitrogen remains abso-

lutely constant, and creatine nitrogen, in contrast to its behavior during small daily injections, changes in parallel with the urinary and urea nitrogen, except for 1 day, May 1 to May 2. Ammonia nitrogen shows no striking effect and the rest nitrogen shows a variable effect. As with repeated injections the nitrogen retained is obviously only a part of



the amino acid nitrogen which, without the influence of the hormone, would have been deaminated and converted to urea. Rest nitrogen is made up in part also of amino acid nitrogen. The stimulus to growth of the accessory structures of the sexual apparatus has laid claim to the amino acids before they could be deaminated or excreted.

In view of this discrepancy in the action of creatine after continuous daily injections and a single large injection, and the fact that these castrate dogs were excreting creatine, an abnormality possessed by castrates, according to Read ('21) but disputed by Shen ('25), it seemed desirable to eliminate the possibility that the excreted creatine might be of exogenous origin, since beef heart contains considerable creatine. Now dog 3, prior to castration had been placed on a diet similar in every respect to that of dog 1 and dog 2 (table 2) and analysis of its urine at the time indicated an abnormal creatine to creatinine ratio of 1.29. Therefore, dog 3 was placed on a creatine free diet, Cowgill's diet for dogs ('23)

TABLE 8

Dog 3. Creatine and creatinine excretion (Cowgill's diet[2])

Date (1935)	5/7	5/8	5/9[1]	5/10	5/11	5/12
Gm. creatine N	0.016	0.010	0.014	0.026	0.007	0.026
Gm. creatinine N	0.204	0.198	0.191	0.197	0.198	0.198

Date (1935)	5/13	5/14	5/15	5/16	5/17	
Gm. creatine N	0.003	0.007	0.008	0.002	0.006	
Gm creatinine N	0.197	0.194	0.200	0.195	0.205	

[1] Injected 9.4 cc. of preparation 67 at 8.00 A.M.

[2] Creatine/creatinine = 1.29 on beef heart diet.

(table 3), with casein the sole source of protein. A study of the nitrogen metabolism before and after the injection of 9.4 cc. (235 to 319 B.U.) of preparation 67, yielded results the same as for dog 2 with the one exception that only traces of creatine could be detected both before and after the injection (table 8).

DISCUSSION

Although in the interest of brevity the complete data have not been presented, the summaries and typical experiments shown adequately explain the results of this investigation.

It is to be noted that the nitrogen retained is never completely excreted. In fact dog 1, the thin castrate, during only

one period, 5 days, in the four experiments, goes into a negative balance. Dog 2, on the other hand, always goes into a negative balance after cessation of injections and gradually returns to equilibrium. Undoubtedly the nitrogen is retained as circulating (or reserve) protein, but it seems difficult to understand why dog 2 should excrete part of the retained nitrogen and dog 1 none, unless we assume that in the latter at least part of the retained nitrogen has been converted to permanent structures.

The difference between dogs 1 and 2 appears to be similar in some respects to that between a pregnant dog in the late weeks of gestation and a non-pregnant dog. Fed high protein both dogs retain nitrogen; when returned to low protein the non-pregnant dog excretes more than the pregnant. A closely parallel situation is found also in menstruation followed by pregnancy (Murlin, '10) where the retention of nitrogen under the influence of oestrin is followed by a minus balance in the early weeks of gestation. Oestrin is no longer effective to produce retention. In the present experiments male hormone causes retention in both dogs, but dog 2 does not retain so tenaciously as dog 1. Dog 2, being fat, suggests a relative pituitary deficiency. Injection of the globulin fraction of the Bugbee extract of anterior pituitary at the end of male hormone injections in the case of dog 2, certainly would have converted the minus balance periods into positive (Gaebler, '33). Gaebler, in fact, finds that the effect of the growth hormone in dogs varies much as the effect of the male hormone does in this study. Sometimes the retention is permanent, at other times temporary, suggesting that some additional factor is necessary to cause conversion of reserve protein into cell structures.

The significant effect on the urinary constituents is the paralleling of the changes in the urinary nitrogen by the urea nitrogen, which was to be expected in decreased catabolism of exogenous protein. The outer urinary constituents do not show any striking differences except possibly creatine.

At first it seemed that the finding of appreciable amounts of creatine in the urine of these castrate dogs confirmed the findings of Read ('21). An investigation, however, as to the source of the creatine excreted indicated that it came from the diet. When the diet did not contain creatine the urine also was free of creatine. Our results on the creatine metabolism of castrates confirm those of Shen ('25) rather than Read ('21).

The creatinine output at no time was affected and in fact remained remarkably constant which cannot be reconciled with the findings of Schrire and Zwarenstein ('32 a and b).

The ammonia nitrogen was always normal with a slight variance which is not significant.

Comparison of the results obtained on the nitrogen retention after the administration of repeated doses and single large doses, indicates clearly that the former is the more efficient method. The administration of a dose of 9.1 cc. of preparation 67 produced in dog 2 a maximum retention of 15.5 per cent, while the injection of ½ cc. of preparation X, twice per day, produced a maximum nitrogen retention of 17 per cent in less than 5 days. Therefore the same effect was produced with less than half as much hormone. There is also a point beyond which increasing the amount of the hormone does not increase the amount of nitrogen retained.

The different results obtained in the heat production of the two dogs can be explained at present only by the difference in their tendencies to become fat. Dog 2, weighing around 22 kg., has a basal heat production of 24 Cal./sq.m./hr., while dog 1, weighing around 12 kg. has a basal of 29 Cal./sq.m./hr., i.e., 20 per cent greater. Injection of male hormone compensates for castration in the fat dog more than in the thin. It must be borne in mind, however, that the increase in heat production produced in dog 2, and this only after several days of repeated injections, is only about 10 per cent. It is significant, therefore, that while the male sex hormone produces a change in the protein and fat metabolism, and a decrease in carbohydrate metabolism in the fat castrate dog, it is not a calorigenic agent of importance.

The difference noted in the energy metabolism of the two dogs and the protein metabolism—the fact that dog 1 does not excrete the nitrogen retained while dog 2 excretes part of its retained nitrogen—suggests the participation of some other endocrine organ.

Furthermore, the striking similarity of our results on nitrogen metabolism, but not on energy metabolism, after the injection of male sex hormone, with those of Teel and Cushing ('30) and Gaebler ('33) after the injection of anterior pituitary growth hormone, suggests a metabolic interrelationship of the gonads and the anterior pituitary.

Both dogs always increased in weight—about ½ kg. dog 1; about 1 kg. dog 2—with a gradual return to preinjection level. The increased body protein is not sufficient to explain these increases. It is possibly due to a gradual hydration and a subsequent dehydration, for no marked thirst or diuresis was noted.

The male sex hormone prepared from urine has not as yet been proven to be chemically or physiologically identical with that produced by the extraction of testicular tissue. Prevailing evidence indicates that not only are the substances produced from the two sources different chemically, but also physiologically. Butenandt ('31, '34) has prepared male sex hormone from urine in pure crystalline form and designated it androsterone. Ruzicka ('34) has prepared an identical product synthetically from cholesterol. Ogata and Hirano ('34) have prepared from boars' testes pure crystalline material which is chemically different from androsterone. Koch and Galagher ('34) report that while the product from urine is stable toward heating with alkali, the product from beef testes is destroyed. Dingemanse, Freud and Laqueur ('35) reported definitely different effects of the two products on the amount of regeneration of prostate and seminal vesicles of castrate rats. Kozelka and Galagher ('34) and Willier, Galagher and Koch ('35) report that extracts of bull testes injected into chick embryos produced no change, while urinary extracts produced normal ovarian tissue in the cortical portion of the left gonad.

Early in our investigations it occurred to us that probably the hormone prepared from the testicular tissue was different and would produce a calorigenic effect. Preliminary experiments suggest this possibility.

ADDENDUM

David et al. ('35) in a preliminary note, report the isolation of pure crystalline hormone from testes (testosteron) which is six times as potent as pure crystalline material from urine (androsterone) and possesses different chemical as well as physiological properties.

CONCLUSIONS

Male hormone as prepared from urine produces the following definite results in adult male castrate dogs.

A marked drop in urinary nitrogen with a subsequent increase which are both due to changes in urea nitrogen. Other constituents show no significant changes. Creatine is not excreted by castrate dogs if on a creatine-free diet.

The decrease of urinary nitrogen does not begin until the first day after the injection. After reaching a maximum, further injections only maintain the decreased protein catabolism at this level.

It is more efficient to use small repeated doses than single large doses.

The heat production of thin castrate dogs is not affected while that of a fat castrate dog is raised slightly, 10 per cent, after repeated injections.

The fat metabolism is increased, the protein metabolism decreased and the carbohydrate metabolism is not affected in the thin dog, but is decreased in the fat dog.

The weights of the dogs show a significant but not large increase during injection, and a prompt return to the pre-injection level thereafter.

LITERATURE CITED

BUTENANDT, A. 1931 Ueber die chemische Untersuchung der Sexualhormon. Zeitschr. angew Chem., Bd. 44, S. 905.

BUTENANDT, A., AND K. TSCHERNING 1934 Ueber Androsteron, ein Krystallisiertes mannliches Sexualhormon. I. Zeitschr. physiol. Chem., Bd. 229, S. 167.

COWGILL, G. R. 1928 An improved procedure for metabolism experiments. J. Biol. Chem., vol. 56, p. 725.

COWGILL, G. R., AND D. L. DRABKIN 1927 Determination of a formula for the surface area of the dog together with a consideration of formulae available for other species. Am. J. Physiol., vol. 81, p. 36.

DAVID, K., E. DINGEMANSE, J. FREUD AND E. LAQUEUR 1935 Über krystallinisches männliches Hormon aus Hoden (Testosteron), wirksamer als aus Harn oder aus Cholesterin bereitetes Androsteron. Z. Physiol. Chem., Bd. 233, S. 281.

DINGEMANSE, E., J. FREUD AND E. LAQUEUR 1935 Differences between male hormone extracts from urine and testes. Nature, vol. 135, p. 184.

FOLIN, O. 1914 On the determination of creatinine and creatine in urine. J. Biol. Chem., vol. 17, p. 469.

FUNK, C., B. HARROW AND A. LEJWA 1930 The male hormone. Am. J. Physiol., vol. 92, p. 440.

GAEBLER, O. H. 1933 Some effects of anterior pituitary extracts on nitrogen metabolism, water balance and energy metabolism. J. Exp. Med., vol. 57, p. 349.

GALAGHER, T. F., AND F. C. KOCH 1934 The effect of alkali on the testicular hormone. J. Biol. Chem., vol. 104, p. 611.

KOCH, F. C. 1932 Sex and internal secretions (E. Allen). Williams and Wilkins Co., Baltimore, p. 372.

KORENCHEVSKY, V. 1925 The sexual glands and metabolism. I. Influence of castration in nitrogen and gaseous metabolism. Brit. J. Exp. Path., vol. 6, p. 21.

KOZELKA, A. W., AND T. F. GALAGHER 1934 Effect of male hormone extracts, theelin and theelol on the chick embryo. Proc. Soc. Exp. Biol. and Med., vol. 31, p. 1143.

LUSK, G. 1924 Animal calorimetry. Analysis of the oxidation of mixtures of carbohydrate and fat. J. Biol. Chem., vol. 59, p. 41.

———— 1928 The Science of Nutrition. W. B. Saunders Co., Philadelphia, 4th ed., p. 65.

MCCULLAGH, E. P., D. R. MCCULLAGH AND N. F. HICKEN 1933 Diagnosis and treatment of hypogonadism in the male. Endocrin., vol. 17, p. 49.

MURLIN, J. R. 1910 Metabolism of development. II. Nitrogen balance during pregnancy and menstruation of the dog. Am. J. Physiol., vol. 27, p. 177.

OGATA, A., AND S. HIRANO 1934 The male hormone. VI. Male hormone from boar's testes. 2. A new crystal male hormone. J. Pharm. Soc. Japan, vol. 54, pp. 1010–1019. (English, pp. 199–211).

READ, B. E. 1921 The metabolism of the eunuch. J. Biol. Chem., vol. 46, p. 281.

RUZICKA, L., M. W. GOLDBERG, J. MEYER, H. BRUNGGER AND E. EICHENBERGER 1934 Zur Kenntnis der Sexualhormon. II. Ueber die Synthese des Testikelhormons (Androsteron) und Stereoisomerer desselben durch Abbau hydrierter Sterine. Helv. Chim. Acta, vol. 17, p. 1395.

SCHRIRE, J., AND H. ZWARENSTEIN 1932 a The influence of the gonads on protein metabolism. Urinary creatinine after castration. Biochem. J., vol. 26, pp. 118–121.

———— 1932 b The influence of the gonads on protein metabolism. II. The effect of injections of saline suspensions of testes and testicular extracts on urinary creatinine after castration. Biochem. J., vol. 26, p. 1886.

SHEN, T. C. 1925 Chemical studies on urine of eunuchs. Proc. Soc. Exp. Biol. and Med., vol. 22, p. 408.

TEEL, H. M., AND H. CUSHING 1930 Studies in the physiological properties of the growth promoting extracts of the anterior pituitary. Endocrin., vol. 14, p. 157.

VAN SLYKE, D. D., AND G. E. CULLEN 1916 The determination of urea by the urease method. J. Biol. Chem., vol. 24, p. 117.

WILLIER, B. H., T. F. GALAGHER AND F. C. KOCH 1935 The action of male and female hormones upon the reproduction glands and ducts of the chick embryo. Proc. Am. Soc. Biol. Chem., vol. XXIX, p. xcix.

A Correction to the Paper Entitled: THE FOOD VALUE OF ETHYL ALCOHOL

H. H. MITCHELL

In the paper entitled: "The food value of ethyl alcohol" that appeared in The Journal of Nutrition for September, 1935 (vol. 10, pp. 311–335), reference was made (p. 330) to a determination of the specific dynamic effect of starch when fed to rats reported by Kriss, Forbes and Miller in a paper that appeared in a previous number of the journal (vol. 8, pp. 509–534). Believing that the specific dynamic effect of starch (and of the other nutrients fed in these experiments) had been computed from heat productions which had been reduced in toto to a constant rat weight of 100 gm., the values of Kriss, Forbes and Miller were recomputed on the assumption that only the basal heat varies directly with the body surface.

Since the publication of the alcohol article, Doctor Forbes has called the author's attention to the fact that the specific dynamic effects were in fact computed from heat production values corrected for differences in body size only with reference to the basal fraction of heat. Hence, the recomputed values of the author, appearing in the text and in footnote 3 on page 331, are in error.

The author regrets exceedingly that he was misled by the brief description of the method of weight correction used by Kriss, Forbes and Miller, as given in their article (p. 514) and in the immediately following one by Forbes, Kriss and Miller (pp. 543 and 545), and by the manner in which the corrected heat production values were expressed, i.e., as hourly heat productions "per 100 gm. of empty body weight."

It need only be said in addition that neither the results of the alcohol experiments nor the conclusions derived from them are in any way impaired by this error.